

JC10 Rec'd PCT/PTO 31 DEC 2007

FORM PTO-1350 (REV 9-2001)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER GJE-83	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 10/019906	
INTERNATIONAL APPLICATION NO. PCT/EP00/06887		INTERNATIONAL FILING DATE 19 July 2000		PRIORITY DATE CLAIMED 19 July 1999	
TITLE OF INVENTION A Method For Amplifying Low Abundance Nucleic Acid Sequences And Means For Performing Said Method					
APPLICANT(S) FOR DO/EO/US Peter Richardson and Peter Cox					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> has been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto.</p> <p style="margin-left: 20px;">b. <input checked="" type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 20px;">d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) <u>(unsigned)</u>.</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11 to 20 below concern document(s) or information included:</p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 37 CFR 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input type="checkbox"/> Other items or information: New page 96 (Abstract of the Disclosure)</p>					

U.S. APPLICATION NO. (if known, see 37 CFR 1.53) 10/019906		INTERNATIONAL APPLICATION NO. PCT/EP00/06887		ATTORNEY'S DOCKET NUMBER GJE-83	
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21. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. \$1040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; text-align: right;">\$890.00</td> <td style="width: 50%;"></td> </tr> </table>		\$890.00	
\$890.00							

Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).					
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CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	
Total claims	<u>138</u> - 20 =	<u>118</u>	x \$18.00	\$2,124.00	
Independent claims	<u>13</u> - 3 =	<u>10</u>	x \$84.00	\$840.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ \$270.00	\$0.00
TOTAL OF ABOVE CALCULATIONS =				\$3,854.00	

<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+	
SUBTOTAL =				\$3,854.00	

Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).					
TOTAL NATIONAL FEE =				\$3,854.00	

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +					
TOTAL FEES ENCLOSED =				\$3,854.00	

	Amount to be refunded:	\$
	charged:	\$

a. ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 19-0065 in the amount of \$ 3,854.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0065. A duplicate copy of this sheet is enclosed.

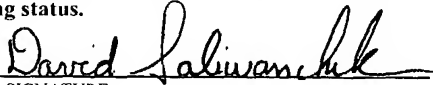
d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

CORRESPONDENCE ADDRESS:

CUSTOMER NUMBER
23,557

December 31, 2001
DATE


 SIGNATURE
 David R. Saliwanchik
 NAME
 31,794
 REGISTRATION NUMBER

10/019906

10/019906

531 Rec'd PCT/PTC 31 DEC 2001

December 31, 2001

Patent Application

Docket No. GJE-83

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Peter Richardson and Peter Cox
Docket No. : GJE-83
For : A Method for Amplifying Low Abundance Nucleic Acid Sequences and
Means For Performing Said Method

Box PCT

Assistant Commissioner for Patents

Washington, D.C. 20231

PRELIMINARY AMENDMENT

It is respectfully requested that the above-referenced patent application be amended to
read as follows:

In the Specification

Please add the following paragraph at page 1, after the title:

Cross Reference to a Related Application

This application is a National Stage Application of International Application
Number PCT/EP00/06887, published, pursuant to PCT Article 21(2), in English,
which claims priority to U.S. Serial No. 60/144,666; filed July 19, 1999.

After page 95: Please insert as new page 96 the attached Abstract of the Disclosure.

In the Claims

Please amend the following claims to read as follows:

Claim 3 (amended):

3. The method of claim 1, wherein the amplification reaction of step c) includes the steps of:

- (i) obtaining single stranded DNA molecules at a temperature between 85°C and 97°C;
- (ii) annealing the single stranded DNA molecules at a temperature between 55°C and 65°C; in the presence of a concentration of magnesium ranging from 1.5-6 mM;
- (iii) elongating the annealed DNA molecules at a temperature between 70°C and 75°C in the presence of a concentration of 4.5 mM Magnesium; and
- (iv) reiterating steps (i) to (iii) for the desired number of cycles.

Claim 4 (amended):

4. The method of claim 1, wherein the amplification of said first and second cDNA strands carried out in step c) comprises between 30 and 50 amplification cycles.

Claim 5 (amended):

5. The method according to claim 1, wherein each amplification reaction cycle of step e) comprises the following steps of:

- (i) obtaining single stranded DNA molecules by incubating the sample at a temperature between 85°C and 95°C;
- (ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature between 55°C and 75°C;

- (iii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature between 65° C and 75° C; and
- (iv) reiterating steps (i) through (iii) for the number of cycles desired.

Claim 6 (amended):

6. The method of claim 1, wherein the further set of amplification reaction cycles carried out in step e) comprises between 10 and 40 cycles.

Claim 7 (amended):

7. The method of claim 5, wherein the amplification reaction of step e) is performed in the presence of magnesium at a concentration from 1.5 to 4.5 mM.

Claim 8 (amended):

8. The method according to claim 1, wherein said method comprises an additional amplification step following step f) which comprises submitting at least a part of the high molecular weight DNA molecules separated at step f) to a further amplification reaction using at least a portion of the first heel sequence and at least a portion of the second heel sequence as the primers.

Claim 9 (amended):

- 9. The method of claim 1, wherein step g) comprises any of the following methods:
 - (i) detection of the sequences of interest with specific oligonucleotide probes;
 - (ii) amplification of the sequences of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector;
and

(iv) in vitro RNA transcription, either for hybridization assays or for further reverse transcription optionally using unlabelled or labeled substrate followed by gene specific PCR or hybridization.

Claim 10 (amended):

10. The method according to claim 1, wherein the first heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample or to the first strand cDNA molecules obtained at step a);

(ii) an oligo dT sequence of 15 to 25 nucleotides in length; and

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof,
wherein substantially every possible variable sequence combination is found in said first heeled primer population.

Claim 12 (amended):

12. The method according to claim 1, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a);

(ii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of 4 to 7 nucleotides is found in said second heeled primer population; and

(iii) a second variable nucleotide sequence that was calculated to hybridize on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

Claim 14 (amended):

14. The method according to claim 12, wherein said second heeled primer comprises a RNA polymerase binding site such as the T7 promoter.

Claim 17 (amended):

17. The method of claim 15, wherein the amplification reaction of step c) includes the following steps of:

(i) obtaining single stranded DNA molecules at a temperature between 85°C and 97°C;

(ii) annealing the single stranded DNA molecules at a temperature between 45°C and 65°C;

(iii) elongating the annealed DNA molecules at a temperature between 70°C and 75°C in the presence of a concentration of 4.5 mM Magnesium; and

- (iv) reiterating steps (i) to (iii) for the desired number of cycles.

Claim 18 (amended):

18. The method of claim 15, wherein the amplification of said first and second cDNA strands carried out in step c) comprises between 30 and 50 amplification cycles.

Claim 19 (amended):

19. The method according to claim 15, wherein the first heeled primer population consists of a population of nucleic acids comprising, from 5' end to 3' end:

- (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;
 - (ii) an oligo dT sequence of 15 to 25 nucleotides in length; and
 - (iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof,
- wherein substantially every possible variable sequence combination is found in said first heeled primer population.

Claim 20 (amended):

20. The method according to claim 19, wherein the variable sequence of 2 to 4 nucleotides is selected from the following variable nucleotide sequence: 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from the group consisting of A, T, C and G.

Claim 21 (amended):

21. The method according to claim 15, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a) and wherein the heel sequence includes the nucleotide sequence of a rare cleavage site in particular a rare restriction site located at its 3' end;

(ii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of 4 to 7 nucleotides is found in said second heeled primer population; and

(iii) a second variable nucleotide sequence that hybridizes on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

Claim 22 (amended):

22. The method according to claim 21, wherein the heel sequence consists of the nucleic acid sequence 5'-CTGCATCTATCTAGTACGCGT-3'.

Claim 23 (amended):

23. The method according to claim 21, wherein said second variable sequence is chosen from the group of sequences consisting of 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3', such that each of said second variable sequence is found in said second heeled primer population.

Claim 24 (amended):

24. The method according to claim 15, wherein the first heeled primer includes the sequence of a rare cleavage site in particular a rare restriction site located at the 3' end of its heel sequence.

Claim 25 (amended):

25. The method according to claim 24, wherein the cleavage site of said first heeled primer is identical to the cleavage site of the second heeled primer.

Claim 26 (amended):

26. The method according to claim 24, wherein the cleavage site of said first heeled primer is different from the cleavage site of the second heeled primer.

Claim 28 (amended):

28. The method according to claim 15, wherein each amplification reaction cycle of step f) comprises the following steps of:

- (i) obtaining single stranded DNA molecules by incubating the sample at a temperature between 85°C and 95°C;
- (ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature between 55°C and 75°C;
- (iii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature between 65°C and 75°C; and
- (iv) reiterating steps (i) through (iii) for the desired number of cycles.

Claim 34 (amended):

34. The heeled primer population according to claim 33, wherein the variable sequence of 2 to 4 nucleotides is selected from the following variable nucleotide sequence: 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from the group consisting of A, T, C and G.

Claim 35 (amended):

35. The heeled primer population of claim 33, wherein the heel sequence comprises the sequence of a rare cleavage site in particular a rare restriction site located at its 3' end.

Claim 38 (amended):

38. The heeled primer population of claim 37, wherein the cleavage site in particular a rare restriction site is selected from the group consisting of Not1, BssH1, Nar1, Mlu1, Nru1 and Nae1.

Claim 39 (amended):

39. The heeled primer population according to claim 37, wherein the heel sequence consists of the nucleic acid sequence 5'-CTGCATCTATCTAGTACGCGT-3'.

Claim 40 (amended):

40. The heeled primer population according to claim 37, which further comprises a RNA polymerase promoter site.

Claim 43 (amended):

43. The kit according to claim 41, which further comprises one or several restriction enzymes that recognize the rare cleavage site in particular a rare restriction site sequence present in the heel sequence of the second heeled primer.

Claim 44 (amended):

44. The kit according to claim 41, which further comprises a RNA polymerase.

Claim 45 (amended):

45. A method to increase the number of sequences corresponding to the mRNA species present in a sample, wherein said method comprises the steps of:

- a) reverse transcribing the mRNA species using a first heeled primer population to provide first strand cDNA species;
- b) synthesizing second cDNA strands using a second heeled primer population;
- c) amplifying said first and second cDNA strands resulting from step b) over a number of amplification cycles with said second heeled primer at a concentration ranging between 0.02 to 200 ng per reaction in the following conditions:
 - (i) obtaining single stranded DNA molecules at a temperature between 78°C and 95°C;
 - (ii) optionally annealing said single stranded DNA molecules at a temperature between 40°C and 72°C;
 - (iii) elongating the annealed DNA molecules at a temperature between 65°C and 75°C in the presence of a thermoresistant DNA polymerase;

Claim 50 (amended):

50. The method of claim 45, wherein each amplification reaction cycle of step d) comprises the following steps of:

- (i) obtaining single stranded DNA molecules by incubating the sample at a temperature between 78°C and 95°C;
- (ii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature between 65°C and 75°C;
- (iv) reiterating steps (i) through (iii) for the desired number of reaction cycles.

Claim 51 (amended):

51. The method of claim 45, wherein the amplification reaction of step d) is performed in the presence of 2.5 mM Magnesium concentration.

Claim 52 (amended):

52. The method of claim 45, wherein said method comprises the additional step of:

- f) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at step e).

Claim 53 (amended):

53. The method according to claim 52, wherein said method comprises a further step wherein the DNA molecules obtained at step f) having a length of less than 50 base pairs are discarded from the reaction mixture.

Claim 54 (amended):

54. The method of claim 52, wherein step f) comprises any of the following methods:

- (i) detection of sequences of interest with specific oligonucleotide probes;
- (ii) amplification of sequences of interest with specific oligonucleotide primers;
- (iii) cloning of the DNA molecules obtained in a replication and/or expression vector;

and

(iv) in vitro RNA transcription, either for hybridization assays or for further reverse transcription using unlabeled or labeled primers or substrates followed by gene specific PCR or hybridization.

Claim 55 (amended):

55. The method according to claim 45, wherein the first heeled primer population consists of a population of nucleic acids comprising, from 5' end to 3' end:

- (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;
- (ii) an oligo dT sequence of 15 to 35 nucleotides in length; and
- (iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population.

Claim 56 (amended):

56. The method according to claim 55, wherein the variable sequence of 2 to 4 nucleotides is selected from the following variable nucleotide sequence: 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from the group consisting of A, T, C and G.

Claim 57 (amended):

57. The method according to claim 45, wherein the first heeled primer includes the sequence of a rare cleavage site in particular a rare restriction site located at the 3' end of its heel sequence.

Claim 58 (amended):

58. The method according to claim 45, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

- (i) a heel sequence of 25 to 30 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a);
- (ii) a first variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of 15 to 25 nucleotides is found in said second heeled primer population; and
- (iii) a second variable nucleotide sequence.

Claim 59 (amended):

59. The method of claim 45, wherein the heel sequence of the second heeled primer comprises the sequence of a rare cleavage site in particular a rare restriction site located at the 3' end of its heel sequence.

Claim 61 (amended):

61. The method of claim 45, wherein the heel sequences of the first and second heeled primers comprise the sequence of a rare cleavage site in particular a rare restriction site located at the 3' end of their respective heel sequence.

Claim 62 (amended):

62. The method according to claim 58, wherein the cleavage site of the first heeled primer is identical to the cleavage site of the second heeled primer.

Claim 63 (amended):

63. The method according to claim 61, wherein the cleavage site of the first heeled primer is different from the cleavage site of the second heeled primer.

Claim 64 (amended):

64. The method of claim 60, wherein step e) is followed by the additional steps of:

- g) incubating the DNA molecules obtained at step e) with at least one restriction enzyme that specifically recognizes the cleavage site in particular a rare restriction site included in the heel sequence of the second heeled primer;

- h) diluting the product of step d) to obtain a diluted cDNA solution containing a cDNA concentration which is between about 2 and 100 times inferior to the cDNA concentration of the product of step d);
- i) adding a thermoresistant DNA polymerase to the diluted sample of step h) and performing a further set of amplification reaction cycles without adding any nucleic acid primer; and
- j) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at steps g), h) and i).

Claim 65 (amended):

65. The method according to claim 64, wherein when the heels of the first and second heeled primers each comprise a rare cleavage site in particular a rare restriction site, the method is characterized in that in step g), the DNA molecules are incubated with restriction enzymes recognizing respectively the rare cleavage sites present in the heel sequence of the first and the second heeled primer.

Claim 66 (amended):

66. The method according to claim 64, wherein said method comprises a further step wherein the DNA molecules obtained at step g) having a length of less than 50 base pairs are separated from the reaction mixture.

specified, and (c) a mixture of the primers (a) and (b), wherein the total concentration of primers ranges between 0.02 and 200 ng per reaction in the following conditions:

(vii) obtaining single stranded DNA molecules at a temperature between 80°C and 95°C;

(viii) adding a thermoresistant DNA polymerase to the single stranded DNA molecules obtained at step (vii);

(ix) annealing and elongating the single stranded DNA molecules at a temperature between 65°C and 75°C; and

(x) carrying out steps (vii) and (ix) for a desired number of cycles.

Claim 73 (amended):

73. The method of claim 69, wherein the amplification steps b) and c) are performed in the presence of a concentration of Magnesium between 2 and 5 mM.

Claim 74 (amended):

74. The method of claim 73, wherein the amplification step d) is performed in the presence of a concentration of Magnesium between 2 and 3 mM.

Claim 76 (amended):

76. The method of claim 75, wherein the respective concentration of primers ranges from 0.02 to 90 ng.

Claim 80 (amended):

80. The method of claim 70, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium between 1.5 and 4 mM.

Claim 81 (amended):

81. The method of claim 80, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium between 1.6 and 2.5 mM.

Claim 82 (amended):

82. The method of claim 80, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium of 2.0 mM.

Claim 85 (amended):

85. The method of claim 69, wherein step b) of synthesizing second cDNA strands is performed in the presence of a Magnesium concentration from 3 to 5 mM.

Claim 86 (amended):

86. The method of claim 85 wherein the Magnesium concentration is 4.5 mM.

Claim 87 (amended):

87. The method of claim 69, wherein step b) of synthesizing second cDNA strands comprises the steps of:

- (i) adding the primers to the cDNA product obtained at step a);

- (ii) obtaining single stranded DNA molecules at a temperature between 80°C and 95°C;
- (iii) adding the DNA polymerase and the proof reading enzyme to the mixture obtained at step (ii);
- (iv) maintaining the temperature of the mixture at approximately 94°C during a period of time between 30 sec to 5 min;
- (v) annealing said single stranded DNA at a temperature between 40°C and 72°C;
- (vi) elongating the annealed DNA molecules at a temperature between 60°C and 75°C.

Claim 88 (amended):

88. The method of claim 69, wherein step c) of synthesizing second cDNA strands comprises the steps of:

- (i) optionally obtaining single stranded DNA molecules at a temperature between 80°C and 95°C in the presence of a thermoresistant DNA polymerase;
- (ii) annealing the single stranded DNA molecules obtained at step (i) with the second heeled primer population at a temperature between 40°C and 60°C;
- (iii) elongating the annealed DNA molecules at a temperature between 65°C and 75°C;
- (iv) repeating steps (ii) to (iii) for a desired number of cycles.

wherein substantially every possible variable sequence combination is found in said first heeled primer population.

Claim 94 (amended):

94. The method according to claim 93, wherein the variable sequence of 2 to 4 nucleotides is selected from the following variable nucleotide sequence: 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from the group consisting of A, T, C and G.

Claim 95 (amended):

95. The method according to claim 69, wherein the first heeled primer includes the sequence of a rare cleavage site in particular a rare restriction site.

Claim 96 (amended):

96. The method according to claim 95, wherein the rare cleavage site is located at the 5' end of the heel sequence of said first heeled primer.

Claim 97 (amended):

97. The method according to claim 95, wherein the rare cleavage site is located at the 3' end of the heel sequence of said first heeled primer.

Claim 98 (amended):

98. The method according to claim 69, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

(i) a heel sequence of 25 to 75 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a); and

(ii) a variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of 15 to 25 nucleotides is found in said second heeled primer population.

Claim 102 (amended):

102. The method of claim 99, wherein the heel sequence of the second heel primer is from 25 to 35 nucleotides in length.

Claim 103 (amended):

103. The method of claim 98 wherein the heel sequence of the second heeled primer is from 45 to 75 nucleotides in length and comprises a RNA polymerase binding site.

Claim 104 (amended):

104. The method of claim 99, wherein the heel sequence of the second heeled primer is from 45 and 75 nucleotides in length and comprises a RNA polymerase binding site located at the 3' end of the heel sequence.

Claim 105 (amended):

105. The method according to claim 99, wherein step e) is followed by the additional steps of:

g) incubating the DNA molecules obtained at step e) with at least one restriction enzyme that specifically recognizes the cleavage site in particular a rare restriction site included in the heel sequence of the second heeled primer;

h) diluting the product obtained at the end of steps d) and g) to obtain a diluted cDNA solution containing a cDNA concentration which is between 2 and 100 times inferior to the cDNA concentration of the product of step d) or g);

i) adding a thermoresistant DNA polymerase to the diluted sample of step h) and performing a further set of amplification reaction cycles without adding any nucleic acid primer; and

j) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at steps g), h) and i).

Claim 106 (amended):

106. The method of claim 105, wherein when the heels of the first and second heeled primers each comprise a rare cleavage site in particular a rare restriction site, the method is characterized in that in step g), the DNA molecules are incubated with restriction enzymes recognizing respectively the rare cleavage sites present in the heel sequence of the first and the second heeled primer.

Claim 107 (amended):

107. The method according to claim 105, wherein said method comprises a further step wherein the DNA molecules obtained at steps e) and g) having a length of less than 50 base pairs are separated from the reaction mixture.

Claim 113 (amended):

113. The heeled primer population of claim 111, wherein the heel sequence comprises the sequence of a rare cleavage site in particular a rare restriction site.

Claim 117 (amended):

117. The heeled primer population of claim 111, wherein the heel sequence has a GC content between 50 and 80%.

Claim 119 (amended):

119. The heeled primer population of claim 118, wherein each primer further comprises:

(iii) a second variable nucleotide sequence that hybridizes on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

Claim 122 (amended):

122. The heeled primer population of claim 121, wherein the sequence of a rare cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence.

Claim 124 (amended):

124. The heeled primer population of claim 121; wherein the cleavage site in particular a rare restriction site is selected from the group consisting of Not1, Bssh11, Nar1, Mlul, Nrul and Nael.

Claim 125 (amended):

125. The heeled primer population of claim 111, wherein the heel sequence has a GC content between 50 and 70%.

Claim 126 (amended):

126. The heeled primer population of claim 115 wherein the heel sequence is from 25 to 35 nucleotides in length.

Claim 127 (amended):

127. The heeled primer population of claim 115 wherein the heel sequence is from 45 to 75 nucleotides in length and comprises a RNA polymerase binding site.

Claim 129 (amended):

129. A kit for the amplification of the mRNA species present in a sample, wherein said kit comprises:

a first heeled primer population comprising:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 35 nucleotides in length; and

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population; and

a second heeled primer population comprising:

- (i) a heel sequence of 25 to 75 nucleotides in length which is not complementary to the mRNA molecules or with first strand cDNA molecules present in a sample; and
- (ii) a first variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of 15 to 25 nucleotides is found in said second heeled primer population.

Claim 130 (amended):

130. The kit of claim 129, which further comprises:

- (iii) a first primer selected from the group consisting of (a) the heel sequence of the first heeled primer and (b) a primer comprising at least 15 consecutive nucleotides of the heel sequence of the first heeled primer;
- (iv) a second primer selected from the group consisting of (a) the heel sequence of the second heeled primer and (b) a primer comprising at least 15 consecutive nucleotides of the heel sequence of the second heeled primer.

Claim 132 (amended):

132. The kit of claim 130, wherein the sequences of the first and second primers are identical.

Claim 133 (amended):

133. The kit according to claim 129, which further comprises a restriction enzyme that recognizes the rare cleavage site in particular a rare restriction site sequence present in the heel sequence of the second heeled primer.

Claim 134 (amended):

134. The kit according to claim 129, which further comprises a restriction enzyme that recognizes the rare cleavage site in particular a rare restriction site sequence present in the heel sequence of the first heeled primer.

Claim 135 (amended):

135. The kit according to claim 129, which further comprises a RNA polymerase.

Claim 136 (amended):

136. A method to increase the number of nucleotide sequences corresponding to the mRNA species present in a low quantity in a sample, comprising:

- a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;
- b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population;
- c) amplifying said first and second cDNA strands resulting from step b) over a number of amplification cycles with the aid of a thermoresistant DNA polymerase(s) with:

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer,

wherein said method is characterized in that it comprises the steps of:

- d) increasing the proportion of high molecular weight DNA molecules,
- e) using or analyzing specific nucleic acid sequences present in the high molecular weight DNA molecules.

Claim 137 (amended):

137. A method to increase the number of nucleic acid sequences corresponding to the mRNA species present in a low quantity in a sample, wherein said method comprises the steps of:

a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;

b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population, wherein each of the primers of said first, and/or second heeled primer population optionally contains a rare cleavage site in particular a rare restriction site located at the 3' end of its heel sequence;

c) amplifying the first and second cDNA strands resulting from step b) over a number of amplification cycles with:

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer;

d) cutting any large DNA molecules and preventing bridge formation in subsequent steps by suppressing the heel portions of at least one said first or second heeled primer; and

e) increasing the amount of long double strand products with sequences more 5' from the original mRNA.

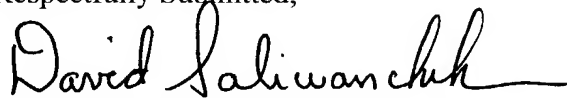
Remarks

Claims 3-10, 12, 14, 17-26, 28-32, 34, 35, 38-41, 43-45, 47-59, 61-70, 73, 74, 76, 80-82, 85-88, 90, 92-98, 102-110, 112, 113, 117, 119, 122, 124-127, 129, 130, and 132-137 have been amended to eliminate multiple dependencies, correct typographical errors, and to lend greater clarity to the claimed subject matter.

No new matter has been added by these amendments.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

Respectfully Submitted,



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DRS/la

Attachment: Marked-up Version of Amended Claims
New page 96 (Abstract of the Disclosure)

Marked-up Version of Amended Claims

Claim 3 (amended):

3. The method of [claims 1 or 2] claim 1, wherein the amplification reaction of step c) includes the steps of:

(i) obtaining single stranded DNA molecules at a temperature [comprised] between 85°C and 97°C;

(ii) annealing the single stranded DNA molecules at a temperature [comprised] between 55°C and 65°C; in the presence of a concentration of magnesium ranging from 1.5-6 mM[.];

(iii) elongating the annealed DNA molecules at a temperature [comprised] between 70°C and 75°C in the presence of a concentration of 4.5 mM Magnesium; and

(iv) reiterating steps (i) to (iii) for the desired number of cycles.

Claim 4 (amended):

4. The method of [any one of claims 1 to 3] claim 1, wherein the amplification of said first and second cDNA strands carried out in step c) comprises between 30 and 50 amplification cycles.

Claim 5 (amended):

5. The method according to [any one of claims 1 to 4] claim 1, wherein each amplification reaction cycle of step c) comprises the following steps of:

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature [comprised] between 85°C and 95°C;

- (ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature [comprised] between 55°C and 75°C;
- (iii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature [comprised] between 65° C and 75° C; and
- (iv) reiterating steps (i) through (iii) for the [desired] number of cycles desired.

Claim 6 (amended):

6. The method of [any one of claims 1 to 5] claim 1, wherein the further set of amplification reaction cycles carried out in step e) comprises between 10 and 40 cycles.

Claim 7 (amended):

7. The method of claim 5[or 6], wherein the amplification reaction of step e) is performed in the presence of magnesium at a concentration [rang:ng] from [1,5] 1.5 to [4,5] 4.5 mM.

Claim 8 (amended):

8. The method according to [any one of claims 1 to 7] claim 1, wherein said method comprises an additional amplification step following step f) which comprises submitting at least a part of the high molecular weight DNA molecules separated at step f) to a further amplification reaction using at least a portion of the first heel sequence and at least a portion of the second heel sequence as the primers.

Claim 9 (amended):

9. The method of [any one of claims 1 to 8] claim 1, wherein step g) comprises any [one] of the following methods:

- (i) detection of the sequences of interest with specific oligonucleotide probes;
- (ii) amplification of the sequences of interest with specific oligonucleotide primers;
- (iii) cloning of the DNA molecules obtained in a replication and/or expression vector;

[or] and

(iv) in vitro RNA transcription, either for hybridization assays or for further reverse transcription optionally using unlabelled or labeled substrate followed by gene specific PCR or hybridization.

Claim 10 (amended):

10. The method according to [any one of claims 1 to 9] claim 1, wherein the first heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample or to the first strand cDNA molecules obtained at step a);

(ii) an oligo dT sequence of 15 to 25 nucleotides in length; and

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population.

- (ii) annealing the single stranded DNA molecules at a temperature [comprised] between 45°C and 65°C;
- (iii) elongating the annealed DNA molecules at a temperature [comprised] between 70°C and 75°C in the presence of a concentration of 4.5 mM Magnesium; and
- (iv) reiterating steps (i) to (iii) for the desired number of cycles.

Claim 18 (amended):

18. The method of [any one of claims 15 to 17] claim 15, wherein the amplification of said first and second cDNA strands carried out in step c) comprises between 30 and 50 amplification cycles.

Claim 19 (amended):

19. The method according to [any one of claims 15 to 18] claim 15, wherein the first heeled primer population consists of a population of nucleic acids comprising, from 5' end to 3' end:

- (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;
 - (ii) an oligo dT sequence of 15 to 25 nucleotides in length; and
 - (iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof,
- wherein substantially every possible variable sequence combination is found in said first heeled primer population.

Claim 20 (amended):

20. The method according to claim 19, wherein the variable sequence of 2 to 4 nucleotides is selected [among] from the following variable nucleotide sequence: 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from the group consisting of A, T, C [or] and G.

Claim 21 (amended):

21. The method according to [any one of claims 15 to 20] claim 15, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a) and wherein the heel sequence includes the nucleotide sequence of a rare cleavage site in particular a rare restriction site located at its 3' end;

(ii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of 4 to 7 nucleotides is found in said second heeled primer population; and

(iii) a second variable nucleotide sequence that [was calculated to hybridize] hybridizes on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

Claim 22 (amended):

[23] 22. The method according to claim 21, wherein the heel sequence consists of the nucleic acid sequence 5'-CTGCATCTATCTAGTACGCGT-3'.

Claim 23 (amended):

23. The method according to claim 21[or 22], wherein said second variable sequence is chosen from the group of sequences consisting of 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3', such that each of said second variable sequence is found in said second heeled primer population.

Claim 24 (amended):

24. The method according to [any one of claims 15 to 23] claim 15, wherein the first heeled primer includes the sequence of a rare cleavage site in particular a rare restriction site located at the 3' end of its heel sequence.

Claim 25 (amended):

25. The method according to claim 24, wherein the cleavage site [in particular a rare restriction site] of said first heeled primer is identical to the cleavage site [in particular a rare restriction site] of the second heeled primer.

Claim 26 (amended):

26. The method according to claim 24, wherein the cleavage site [in particular a rare restriction site] of said first heeled primer is different from the cleavage site [in particular a rare restriction site] of the second heeled primer.

Claim 28 (amended):

28. The method according to [any one of claims 15 to 27] claim 15, wherein each amplification reaction cycle of step f) comprises the following steps of:

- (i) obtaining single stranded DNA molecules by incubating the sample at a temperature [comprised] between 85°C and 95°C;
- (ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature [comprised] between 55°C and 75°C;
- (iii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature [comprised] between 65°C and 75°C; and
- (iv) reiterating steps (i) through (iii) for the desired number of cycles.

Claim 29 (amended):

29. The method of [any one of claims 15 to 28] claim 15, wherein the further set of amplification reaction cycles carried out in step (f) comprises between 20 and 40 cycles.

Claim 30 (amended):

30. The method of claim 28[or 29], wherein the amplification reaction of step f) is performed in the presence of a 3.5 mM Magnesium concentration.

Claim 31 (amended):

31. The method according to [any one of claims 15 to 30] claim 15, wherein said method comprises a further step wherein the DNA molecules obtained at step f) having a length of less than 50 base pairs are separated from the reaction mixture.

Claim 32 (amended):

32. The method of [any one of claims 15 to 31] claim 15, wherein step g) comprises any [one] of the following methods:

- (i) detection of sequences of interest with specific oligonucleotide probes;
- (ii) amplification of sequences of interest with specific oligonucleotide primers;
- (iii) cloning of the DNA molecules obtained in a replication and/or expression vector;

[or] and

(iv) in vitro RNA transcription, either for hybridization assays or for further reverse transcription using unlabeled or labeled primers or substrates followed by gene specific PCR or hybridization.

Claim 34 (amended):

34. The heeled primer population according to claim 33, wherein the variable sequence of 2 to 4 nucleotides is selected [among] from the following variable nucleotide sequence: 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from the group consisting of A, T, C [or] and G.

Claim 35 (amended):

35. The heeled primer population of claim 33[or 34], wherein the heel sequence comprises the sequence of a rare cleavage site in particular a rare restriction site located at its 3' end.

Claim 38 (amended):

38. The heeled primer population of claim 37, wherein the cleavage site in particular a rare restriction site is selected from the group consisting of Not1, Bssh11, Nar1, Mlul, Nrul and Nael.

Claim 39 (amended):

39. The heeled primer population according to claim 37[or 38], wherein the heel sequence consists of the nucleic acid sequence 5'-CTGCATCTATCTAGTACGCGT-3'.

Claim 40 (amended):

40. The heeled primer population according to [any one of claims 37 to 39] claim 37, which further comprises a RNA polymerase promoter site.

Claim 41 (amended):

41. A kit for the amplification of the mRNA species present in a sample, wherein said kit comprises:

[(i)] a first heeled primer population [according to any one of claims 33 to 36;]
comprising:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 25 nucleotides in length;

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof,

Claim 45 (amended):

45. A method to increase the number of sequences corresponding to the mRNA species present in a sample, wherein said method comprises the steps of:

- a) reverse transcribing the mRNA species using a first heeled primer population to provide first strand cDNA species;
- b) synthesizing second cDNA strands using a second heeled primer population;
- c) amplifying said first and second cDNA strands resulting from step b) over a number of amplification cycles with said second heeled primer at a concentration ranging between 0.02 to 200 ng per reaction in the following conditions:
 - (i) obtaining single stranded DNA molecules at a temperature [comprised] between 78°C and 95°C;
 - (ii) optionally annealing said single stranded DNA molecules at a temperature [comprised] between 40°C and 72°C;
 - (iii) elongating the annealed DNA molecules at a temperature [comprised] between 65°C and 75°C in the presence of a thermoresistant DNA polymerase;
- d) amplifying the DNA molecules resulting from step c) over a number of further amplification cycles with:
 - (i) a first primer comprising the heel sequence of the first heeled primer; and
 - (ii) a second primer comprising the heel sequence of the second heeled primer,wherein the respective concentration of each primer ranges between 10 and 500 ng per reaction, whereby a population of amplified DNA molecules is obtained; and
- e) recovering the population of DNA molecules obtained at step d).

Claim 47 (amended):

47. The method of claim 45[or 46], wherein the amplification reaction of step c) is performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme.

Claim 48 (amended):

48. The method of [any one of claims 45 to 47] claim 45, wherein the amplification reaction of step c) is performed in the presence of a concentration of 4.5 mM Magnesium.

Claim 49 (amended):

49. The method of [any one of claims 45 to 48] claim 45, wherein the further set of amplification reaction cycles carried out in step d) comprises between 30 and 50 cycles.

Claim 50 (amended):

50. The method of [any one of claims 45 to 49] claim 45, wherein each amplification reaction cycle of step d) comprises the following steps of:

- (i) obtaining single stranded DNA molecules by incubating the sample at a temperature [comprised] between 78°C and 95°C;
- (ii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature [comprised] between 65°C and 75°C;
- (iv) reiterating steps (i) through (iii) for the desired number of reaction cycles.

Claim 51 (amended):

51. The method of [any one of claims 45 to 50] claim 45, wherein the amplification reaction of step d) is performed in the presence of 2.5 mM Magnesium concentration.

Claim 52 (amended):

52. The method of [any one of claims 45 to 51] claim 45, wherein said method comprises the additional step of:

f) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at step e).

Claim 53 (amended):

53. The method according to claim 52, wherein said method comprises a further step wherein the DNA molecules obtained at step f) having a length of less than 50 base pairs are discarded from the reaction mixture.

Claim 54 (amended):

54. The method of claim 52[or 53], wherein step f) comprises any [one] of the following methods:

- (i) detection of sequences of interest with specific oligonucleotide probes;
- (ii) amplification of sequences of interest with specific oligonucleotide primers;
- (iii) cloning of the DNA molecules obtained in a replication and/or expression vector;

[or] and

(iv) in vitro RNA transcription, either for hybridization assays or for further reverse transcription using unlabeled or labeled primers or substrates followed by gene specific PCR or hybridization.

Claim 55 (amended):

55. The method according to [any one of claims 45 to 54] claim 45, wherein the first heeled primer population consists of a population of nucleic acids comprising, from 5' end to 3' end:

- (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;
 - (ii) an oligo dT sequence of 15 to 35 nucleotides in length; and
 - (iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof,
- wherein substantially every possible variable sequence combination is found in said first heeled primer population.

Claim 56 (amended):

56. The method according to claim 55, wherein the variable sequence of 2 to 4 nucleotides is selected [among] from the following variable nucleotide sequence: 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from the group consisting of A, T, C [or] and G.

Claim 61 (amended):

61. The method of [any one of claims 45 to 60] claim 45, wherein the heel sequences of the first and second heeled primers comprise the sequence of a rare cleavage site in particular a rare restriction site located at the 3' end of their respective heel sequence.

Claim 62 (amended):

62. The method according to claim 58, wherein the cleavage site [in particular a rare restriction site sequence] of the first heeled primer is identical to the cleavage site [in particular a rare restriction site sequence present in the heel] of the second heeled primer.

Claim 63 (amended):

63. The method according to claim 61, wherein the cleavage site [in particular a rare restriction site sequence] of the first heeled primer is different from the cleavage site [in particular a rare restriction site sequence present in the heel] of the second heeled primer.

Claim 64 (amended):

64. The method of [any one of claims 60 to 63] claim 60, wherein step e) is followed by the additional steps of:

g) incubating the DNA molecules obtained at step e) with at least one restriction enzyme that specifically recognizes the cleavage site in particular a rare restriction site included in the heel sequence of the second heeled primer;

- h) diluting the product of step d) to obtain a diluted cDNA solution containing a cDNA concentration which is between about 2 and 100 times inferior to the cDNA concentration of the product of step d);
- i) adding a thermoresistant DNA polymerase to the diluted sample of step h) and performing a further set of amplification reaction cycles without adding any nucleic acid primer; and
- j) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at steps g), h) and i).

Claim 65 (amended):

65. The method according to claim 64, wherein when the heels of the first and second heeled primers each comprise a rare cleavage site in particular a rare restriction site, the method is characterized in that in step g), the DNA molecules are incubated with restriction enzymes recognizing respectively the rare cleavage [site] sites [in particular a rare restriction sites] present in the heel sequence of the first and the second heeled primer.

Claim 66 (amended):

66. The method according to claim 64[or 65], wherein said method comprises a further step wherein the DNA molecules obtained at step g) having a length of less than 50 base pairs are separated from the reaction mixture.

portion of the heel sequence of the second heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, and (3) a mixture of the primers (1) and (2), wherein the total concentration of primers ranges between 0.02 and 500 ng per reaction in the following conditions:

- (i) adding the primers to the cDNA product obtained at step c);
 - (ii) obtaining single stranded DNA molecules at a temperature [comprised] between 80°C and 95°C;
 - (iii) adding a thermoresistant DNA polymerase;
 - (iv) maintaining the temperature at a range from 80°C to 95°C for a period of time [comprised] between 5 sec to 15 min;
 - (v) annealing said single stranded DNA and elongating the annealed DNA molecules at a temperature [comprised] between 65°C and 75°C;
 - (vi) carrying out steps (iv) and (v) for a desired number of cycles.
- e) recovering the population of DNA molecules obtained at step d).

Claim 70 (amended):

70. The method of claim 69, wherein step d) further comprises the steps of amplifying the DNA molecules obtained at step d) (vi) over a number of amplification cycles with primers selected from the group consisting of (a) a primer comprising a portion of the heel sequence of the first heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, (b) a primer comprising a portion of the heel sequence of the second heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions

specified, and (c) a mixture of the primers (a) and (b), wherein the total concentration of primers ranges between 0.02 and 200 ng per reaction in the following conditions:

(vii) obtaining single stranded DNA molecules at a temperature [comprised] between 80°C and 95°C;

(viii) adding a thermoresistant DNA polymerase to the single stranded DNA molecules obtained at step (vii);

(ix) annealing and elongating the single stranded DNA molecules at a temperature [comprised] between 65°C and 75°C[.]; and

(x) carrying out steps (vii) and (ix) for a desired number of cycles.

Claim 73 (amended):

73. The method of claim 69, wherein the amplification steps b) and c) are performed in the presence of a concentration of Magnesium [comprised] between 2 and 5 mM.

Claim 74 (amended):

74. The method of claim [88] 73, wherein the amplification step d) is performed in the presence of a concentration of Magnesium [comprised] between 2 and 3 mM.

Claim 76 (amended):

76. The method of claim [69] 75, wherein the respective concentration of primers ranges from 0.02 to 90 ng.

Claim 80 (amended):

80. The method of claim 70, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium [comprised] between 1.5 and 4 mM.

Claim 81 (amended):

81. The method of claim 80, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium [comprised] between 1.6 and 2.5 mM.

Claim 82 (amended):

82. The method of claim 80, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium of 2.0 [nM] mM.

Claim 85 (amended):

85. The method of claim 69, wherein step b) of synthesizing second cDNA strands is performed in the presence of a Magnesium concentration [ranging] from 3 to 5 [nM] mM.

Claim 86 (amended):

86. The method of claim 85 wherein the Magnesium concentration is [of] 4.5 mM.

Claim 87 (amended):

87. The method of claim 69[or 70], wherein step b) of synthesizing second cDNA strands comprises the steps of:

- (i) adding the primers to the cDNA product obtained at step a);
- (ii) obtaining single stranded DNA molecules at a temperature [comprised] between 80°C and 95°C;
- (iii) adding the DNA polymerase and the proof reading enzyme to the mixture obtained at step (ii);
- (iv) maintaining the temperature of the mixture at approximately 94°C during a period of time [comprised] between 30 sec to 5 min;
- (v) annealing said single stranded DNA at a temperature [comprised] between 40°C and 72°C;
- (vi) elongating the annealed DNA molecules at a temperature [comprised] between 60°C and 75°C[;].

Claim 88 (amended):

88. The method of claim 69[or 70], wherein step c) of synthesizing second cDNA strands comprises the steps of:

- (i) optionally obtaining single stranded DNA molecules at a temperature [comprised] between 80°C and 95°C in the presence of a thermoresistant DNA polymerase;
- (ii) annealing the single stranded DNA molecules obtained at step (i) with the second heeled primer population at a temperature [comprised] between 40°C and 60°C;

(iii) elongating the annealed DNA molecules at a temperature [comprised] between 65°C and 75°C;

(iv) repeating steps (ii) to (iii) for a desired number of cycles.

Claim 90 (amended):

90. The method of claim 69[or 70], wherein said method comprises the additional step of:

f) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at step e).

Claim 92 (amended):

92. The method of claim 69[or 70], wherein step f) comprises any [one] of the following methods:

- (i) detection of sequences of interest with specific oligonucleotide probes;
- (ii) amplification of sequences of interest with specific oligonucleotide primers;
- (iii) cloning of the DNA molecules obtained in a replication and/or expression vector;

[or] and

(iv) in vitro RNA transcription, either for hybridisation assays or for further reverse transcription using unlabeled or labeled primers or substrates followed by gene specific PCR or hybridisation.

Claim 93 (amended):

93. The method according to [any one of claims 69 to 92] claim 69, wherein the first heeled primer population consists of a population of nucleic acids comprising, from 5' end to 3' end:

- (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;
 - (ii) an oligo dT sequence of 15 to 35 nucleotides in length; and
 - (iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof,
- wherein substantially every possible variable sequence combination is found in said first heeled primer population.

Claim 94 (amended):

94. The method according to claim 93, wherein the variable sequence of 2 to 4 nucleotides is selected [among] from the following variable nucleotide sequence: 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from the group consisting of A, T, C [or] and G.

Claim 95 (amended):

95. The method according to [any one of claims 69 to 94] claim 69, wherein the first heeled primer includes the sequence of a rare cleavage site in particular a rare restriction site.

Claim 103 (amended):

103. The method of claim 98 wherein the heel sequence of the second heeled primer [ranges] is from 45 to 75 nucleotides in length and comprises a RNA polymerase binding site.

Claim 104 (amended):

104. The method of claim 99, wherein the heel sequence of the second heeled primer [ranges] is from 45 and 75 nucleotides in length and comprises a RNA polymerase binding site located at the 3' end of the heel sequence.

Claim 105 (amended):

105. The method according to [any one of claims 99 to 104] claim 99, wherein step e) is followed by the additional steps of:

g) incubating the DNA molecules obtained at step e) with at least one restriction enzyme that specifically recognizes the cleavage site in particular a rare restriction site included in the heel sequence of the second heeled primer;

h) diluting the product obtained at the end of steps d) and g) to obtain a diluted cDNA solution containing a cDNA concentration which is between 2 and 100 times inferior to the cDNA concentration of the product of step d) or g);

i) adding a thermoresistant DNA polymerase to the diluted sample of step h) and performing a further set of amplification reaction cycles without adding any nucleic acid primer; and

j) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at steps g), h) and i).

Claim 106 (amended):

106. The method of claim 105, wherein when the heels of the first and second heeled primers each comprise a rare cleavage site in particular a rare restriction site, the method is characterized in that in step g), the DNA molecules are incubated with restriction enzymes recognizing respectively the rare cleavage [site] sites [in particular a rare restriction sites] present in the heel sequence of the first and the second heeled primer.

Claim 107 (amended):

107. The method according to claim 105[or 106], wherein said method comprises a further step wherein the DNA molecules obtained at steps e) and g) having a length of less than 50 base pairs are separated from the reaction mixture.

Claim 108 (amended):

108. The method according to [any one of claims 105 to 107] claim 105, wherein the number of amplification reaction cycles performed in step i) is [comprised] between 20 and 40.

Claim 109 (amended):

109. The method of [any one of claims 105 to 108] according to claim 105, wherein step j) comprises any [one] of the following methods:

- (i) detection of sequences of interest with specific oligonucleotide probes;
- (ii) amplification of sequences of interest with specific oligonucleotide primers; and
- (iii) cloning of the DNA molecules obtained in a replication and/or expression vector.

Claim 110 (amended):

110. The method according to [any one of claims 69 to 109] claim 69, wherein the heel sequences of the first heeled primer and the second heeled primer are identical.

Claim 112 (amended):

112. The heeled primer population according to claim 111, wherein the variable sequence of 2 to 4 nucleotides is selected [among] from the following variable dinucleotide sequence: 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from the group consisting of A, T, C [or] and G.

Claim 113 (amended):

113. The heeled primer population of claim 111[or 112], wherein the heel sequence comprises the sequence of a rare cleavage site in particular a rare restriction site.

Claim 117 (amended):

117. The heeled primer population of [any one of claims 111 to 116] claim 111, wherein the heel sequence has a GC content [comprised] between 50 and 80%.

Claim 119 (amended):

119. The heeled primer population of claim 118, wherein each primer further comprises:

(iii) a second variable nucleotide sequence that [was calculated to hybridize] hybridizes on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

Claim 122 (amended):

122. The heeled primer population of claim [1201] 121, wherein the sequence of a rare cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence.

Claim 124 (amended):

124. The heeled primer population of claim 121, wherein the cleavage site in particular a rare restriction site is selected from the group consisting of Not1, BssH1, Nar1, Mlu1, Nru1 and Nae1.

Claim 125 (amended):

[124] 125. The heeled primer population of [any one of claims 111 to 124] claim 111, wherein the heel sequence has a GC content [comprised] between 50 and 70%.

Claim 126 (amended):

126. The heeled primer population of claim 115 wherein the heel sequence [ranges] is from 25 to 35 nucleotides in length.

Claim 127 (amended):

127. The heeled primer population of claim 115 wherein the heel sequence [ranges] is from 45 to 75 nucleotides in length and comprises a RNA polymerase binding site.

Claim 129 (amended):

129. A kit for the amplification of the mRNA species present in a sample, wherein said kit comprises:

[(i)] a first heeled primer [according to any one of claims 11 to 116] population comprising:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 35 nucleotides in length; and

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population; and

[(ii)] a second heeled primer population [according to claims 117 to 127] comprising:

(i) a heel sequence of 25 to 75 nucleotides in length which is not complementary to the mRNA molecules or with first strand cDNA molecules present in a sample; and

(ii) a first variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of 15 to 25 nucleotides is found in said second heeled primer population.

Claim 130 (amended):

130. The kit of claim [126] 129, which further comprises:

(iii) a first primer selected from the group consisting of (a) the heel sequence of the first heeled primer and (b) a primer comprising at least 15 consecutive nucleotides of the heel sequence of the first heeled primer[.];

(iv) a second primer selected from the group consisting of (a) the heel sequence of the second heeled primer and (b) a primer comprising at least 15 consecutive nucleotides of the heel sequence of the second heeled primer.[;]

Claim 132 (amended):

132. The kit of claim 130, wherein the sequences of the first [-]and second primers are identical.

Claim 133 (amended):

133. The kit according to [anyone of claims 129 to 132] claim 129, which further comprises a restriction enzyme that recognizes the rare cleavage site in particular a rare restriction site sequence present in the heel sequence of the second heeled primer.

Claim 134 (amended):

134. The kit according to [any one of claims 129 to 133] claim 129, which further comprises a restriction enzyme that recognizes the rare cleavage site in particular a rare restriction site sequence present in the heel sequence of the first heeled primer.

Claim 135 (amended):

135. The kit according to [any one of claims 129 to 134] claim 129, which further comprises a RNA polymerase.

Claim 136 (amended):

136. A method to increase the number of nucleotide sequences corresponding to the mRNA species present in a low quantity in a sample, comprising:

- a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;
- b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population;
- c) amplifying said first and second cDNA strands resulting from step b) over a number of amplification cycles with the aid of a thermoresistant DNA polymerase(s) with:
 - (i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and
 - (ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer,

wherein said method is characterized in that it comprises the steps of:

- d['] increasing the proportion of high molecular weight DNA molecules,
- e['] using or analyzing specific nucleic acid sequences present in the high molecular weight DNA molecules[.].

Claim 137 (amended):

137. A method to increase the number of nucleic acid sequences corresponding to the mRNA species present in a low quantity in a sample, wherein said method comprises the steps of:

- a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;
- b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population, wherein each of the primers of said first, and/or second heeled primer population optionally contains a rare cleavage site in particular a rare restriction site located at the 3' end of its heel sequence;
- c) amplifying the first and second cDNA strands resulting from step b) over a number of amplification cycles with:
 - (i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and
 - (ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer;
- d) cutting any large DNA molecules and preventing bridge formation in subsequent steps by suppressing the heel portions of at least one said first or second heeled primer; and
- e) increasing the amount of long double strand products with sequences more 5' from the original mRNA.

Abstract of the Disclosure

The present invention relates to methods as well as to nucleic acid primers and kits containing the same for performing efficiently an amplification of nucleic acid sequences from a sample, particularly of nucleic acid sequences that are initially poorly represented in said sample.

PTO/PCT Rec'd 31 DEC 2001

**A method for amplifying low abundance nucleic acid
sequences and means for performing said method**

5 **FIELD OF THE INVENTION**

The present invention relates to methods as well as to nucleic acid primers and kits containing the same for performing efficiently an amplification of nucleic acid sequences from a sample, particularly of
10 nucleic acid sequences that are initially poorly represented in said sample.

BACKGROUND OF THE INVENTION

15 DNA sequence information resulting from genome and expressed sequence tag (EST) sequencing projects is expected to provide the basis for further understanding of the control and mode of action of individual, and groups of gene products.

In this respect, analysis and comparison of when, where and to
20 what degree genes are expressed, commonly known as expression profiling, is playing an essential role in the functional characterization of newly identified genes.

Many tissue cellular systems, such as the immune and nervous systems, are composed of highly heterogeneous cell populations. A key
25 factor lies in understanding their physiology, and the role of specific gene products expressed with the ability to examine gene usage in the context of this cellular diversity.

In the past, low throughput and laborious methods such as Northern Blotting and nuclease protection assays were employed to
30 study gene expression.

More recently, various methods have been developed for assessing simultaneously the expression of large numbers of genes.

All these techniques, however, require relatively large amounts of RNA and currently lack the sensitivity to analyze specimens derived
35 from small populations of cells or indeed from an individual cell.

This is compounded by the fact that it is very difficult in the case of many cell types to obtain enough specific cellular material for RNA experimentation. Consequently many areas of investigation are frustrated by lack of starting material.

5 Thus in situ hybridization provides detailed information on the cellular expression pattern of a gene in intact tissue. However, this technique is laborious to perform, and does not allow the analysis of more than a very small number of transcripts in a single preparation, when performed in whole-mounts or tissue sections.

10 The polymerase chain reaction (PCR) has been used successfully to investigate gene expression in cytoplasmic samples, particularly with the nested-primer approach which provides good sensitivity, but restricts the analyses to a small number of closely related genes from specific gene families.

15 Some techniques allow detection of the expression of unrelated genes in a single cell, such as T7 RNA polymerase amplification of mRNA and PCR after prior homopolymeric tailing of the first strand cDNA. However, neither of these approaches have been demonstrated to allow the analysis of more than a small number of genes and are not
20 widely used.

The former is technically difficult, whilst the latter may be biased against long transcripts and often requires subsequent cloning of the amplified products.

25 Alternatively, a method for expression profiling in single cells using 3' end amplification PCR has been developed by Dixon *et al.* (1998, Nucleic acids research, vol. 26 (n°19): 4426-4431). This method comprises a first step wherein mRNA species present in a cell are reversed transcribed using a first heeled primer, thereby providing a population of first strand cDNA species and a second step wherein
30 partial 3' end second cDNA strand populations are synthesized using a second heeled primer population.

Using this technique of amplification, the authors have succeeded in detecting, from a mRNA population contained in the cytoplasm of a single cell, the presence of poorly expressed transcripts
35 in cholinergic interneurons such as the neurokinin type 1 receptor.

DETAILED DESCRIPTION OF THE INVENTION

Three different ways of amplifying low amounts of mRNA present in a sample have been found, each of these methods being described in detail hereafter. Thus, these, ways of amplifying allow in several instances to amplify all mRNA species which are present in the sample of interest. Also included is a detailed description of reagents and oligonucleotide primers required for amplifying low amounts of mRNA

FIRST EMBODIMENT OF THE INVENTION.

The first embodiment of the amplification method takes advantage of the generation high molecular weight DNA molecules are formed following amplification of the cDNA species obtained through reverse transcription of the initial mRNA species present in the sample. The inventors have found that these high molecular weight DNA molecules or bridged products may result from the formation of partially duplexed DNA molecules during the annealing step. These partially duplexed DNA molecules would contain partially complementary sequences that hybridize with one another in the low stringency hybridization conditions used, thus forming bridges between two structurally related or unrelated amplified cDNA molecules contained in the amplification mixture. Repetitive amplification cycles result in large nucleic acid molecules. Furthermore, the first embodiment makes use of the high molecular weight DNA produced by this process to analyze amplified species of interest.

The first embodiment makes use of these findings by providing a process which by favoring an increase in the production of these high molecular weight DNA molecules in particular, allows to amplify mRNA species present in a low quantity in a sample to be analyzed.

More precisely, there is disclosed a method to increase the number of nucleotide sequences corresponding to the mRNA species present in a low quantity in a sample, comprising:

a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;

5 b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population;

c) amplifying said first and second cDNA strands resulting from step b) over a number of amplification cycles with the aid of a thermoresistant DNA polymerase(s) with:

10 (i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer,

15 wherein said method is characterized in that it comprises the steps of:

d') increasing the proportion of high molecular weight DNA molecules,

e') using or analyzing specific nucleic acid sequences present in the high molecular weight DNA molecules,

20

PRIMERS

The term "heeled" primer will be readily understood in the art to be a primer comprising a hybridizing region and a non-hybridizing region, wherein the non-hybridizing region represents the "heel" of the primer.

The first heeled primer is actually a population of individual primer species. The first heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

- 30 (i) a heel sequence of 15 to 22 nucleotides in length which does not hybridize with the mRNA molecules initially present in the sample;
- (ii) an oligo dT sequence of 15 to 25 nucleotides in length;
- 35 (iii) a nucleotide which should not be thymidine (A, C or G); and

(iv) a variable sequence of 2-4 nucleotides in length.

The components described in iii and iv being capable of hybridizing to a mRNA molecule at the 5' end of the poly-A tail thereof, wherein substantially every possible variable sequence combination is found in said first heeled primer population .

In a specific embodiment of the first heeled primer, the variable sequence of 2 to 4 nucleotides is selected among the following variable nucleotides sequence:

5'-(A or G or C)-N₁₋₃-, wherein N is a nucleotide selected from A, T, C or G.

In a preferably advantageous alternative, the first heeled primer may also comprise an RNA polymerase binding site, such as the T7, T3 or SP6 promoter, located between the oligo dT sequence and the heel.

The second heeled primer is also a population of individual primer species. When the first strand cDNA population is contacted with the second heeled primer population under appropriate hybridizing conditions, each cDNA species will hybridize with at least one second heeled primer, (partly because of the selection of nucleotide sequences amongst the second heeled primers), second cDNA strand synthesis then proceeds in a 5' to 3' direction from the hybridized second primer.

The second heeled primer population may comprise primers differing by up to five nucleotide bases (differing in the hybridizing region), the second heeled primer population preferably comprising a number of primers in the range 1000 to 100,000 primers, more preferably in the range 1024 to 65536 primers. In order to achieve this, the primers of the second heeled primer population preferably each comprise a first variable sequence of nucleotides in the range of 4 to 7 nucleotides 3' to the heel and a second variable sequence of at least 5 nucleotides contiguous 3' therewith. As will be appreciated, where there are 5 random nucleotides (which is preferred) there will be 4⁵ (i.e. 1024) possible pentamer sequences.

The second variable sequence of this primer may be selected by sequence analysis of known sequences so as to promote the ability of

the second heeled primer as a whole to hybridize to the transcribed cDNA species. Sequence analysis can be carried out through databases of DNA or RNA sequences. In particular, known sequences of the organism of interest are preferably consulted. The second variable
5 sequence of nucleotides preferably comprises a number of nucleotides in the range 2 to 10 nucleotides. In a particularly preferred embodiment, the second variable sequence of nucleotides may comprise a number of nucleotides equivalent to the number of nucleotides in the first variable sequence of this primer.

10 The second variable nucleotide sequence of the second heeled primers may be constant throughout the population of these primers and it is selected so as to stabilize the primers and to ensure optimal efficiency of hybridization to the target first strand cDNA species.

In a preferred embodiment, the second heeled primer from the
15 population of second primers preferably hybridizes on average once in every 1kb portion of first strand cDNA species. This has been found to produce optimal amplification of mRNA in a sample.

Particularly preferred second variable sequences of nucleotides in the second primers are:

20 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3'

The non hybridizing heel regions of the first and second heeled primers are preferably selected so that they lack the ability to hybridize to mRNA or first strand cDNA. The heel regions, like the hybridizing or
25 variable sequence regions of the second primers, are selected by analysis of known nucleotide sequences. In particularly preferred embodiments, the heel regions preferably comprise sequences absent from the mRNA species in the sample. However, the heel regions may simply comprise sequences absent from the genome of the organism
30 from which the sample is taken. The heel regions preferably comprise a number of nucleotides in the range 15 to 22, more preferably 18 to 20 nucleotides.

Preferably, the heel sequences are chosen among nucleic acid sequences having a GC content of about 50%, or for example from
35 about 43% to about 55% of the heel sequence.

mM and 6 mM magnesium and most preferably about 4.5 mM magnesium.

In the case wherein the concentration of magnesium in the elongation buffer has been adjusted to 4.5 mM, the temperature of
5 annealing between the second heeled primer and the first cDNA strands is of about 50°C and the elongation temperature in the presence of the suitable DNA polymerase is of about 72°C.

The cDNA molecules that are generated at the end of step b) are highly representative of the spectrum of mRNA molecules in a
10 sample, as mRNA species of low abundance are reverse-transcribed to the same level of efficiency as more abundant mRNA species.

Step c):

The amplification reaction of step c) is performed with a pair of
15 oligonucleotide primers that respectively comprise at least a portion of the heel sequence of the first and second heeled primers that are defined above.

The first primer of step c) is preferably the heel of the first heeled primer. The second primer of step c) is preferably the heel of the
20 second heeled primer.

The second primer of step c) may be the same as the second heeled primer and this can be advantageous in reducing the number of reagents needed to perform the first embodiment

A further alternative is to use the second heeled primer as the
25 sole primer.

Preferably, the amplification reaction of step c) is performed using low stringency hybridization conditions. For example, amplification reactions are performed in the presence of a concentration of
30 magnesium generally up to 5 mM, preferably between 4 mM and 5 mM magnesium and most preferably of about 4.5 mM magnesium. With the latter magnesium concentration, each amplification cycle comprises a denaturing step at 92°C, an annealing step at 60°C and an elongation step at 72°C.

Advantageously, the amplification reaction of step c) comprises from 30 to 50 amplification cycles, and most preferably comprises about 40 amplification cycles. However, other cycle numbers could be envisaged. One parameter of the optimum number of cycles required is
5 determined by the polymerase used.

In a first series of further process steps, the cDNA may be submitted to *in vitro* transcription either immediately after step c) if the appropriate concentration of cDNA is present in the sample or after
10 further amplification, such as through step d'), steps d) and e) which is/are discussed in more detail below. In this context, it is essential that at least one of the primers used in step a), b) and/or c) comprises a RNA polymerase binding site such as the T7 RNA polymerase promoter. The RNA generated can then be subjected to further process steps, for
15 instance by being labeled during reverse transcription and hybridized to DNA arrays. Alternatively, cDNA generated in the presence or absence of a labeled substrate can be used in gene-specific PCR experiments.

Step d'):

20 In the preferred embodiment of step d'), step d') is a combination of the following steps d) and e).

Step d):

At the end of step c), the reaction mixture containing the
25 population of amplified DNA molecules which include the "bridged products" can be diluted to obtain a diluted cDNA solution containing a cDNA concentration which is between 2 and 100 x inferior to the cDNA concentration of the product of step c). Preferably the diluted cDNA concentration ranges between about 2 and 100 times less, and most
30 preferably between 40 and 80 times less, than the initial cDNA concentration found in the reaction mixture obtained at the end of step c). This dilution step is essential for performing the further steps of the method as it results in the almost complete elimination of the primers initially added to the amplification mixture. The elimination of most of the
35 primers, which are not part of the original gene sequence to be detected,

reduces the element of randomness which would be introduced in the further amplification steps. This element of randomness arises from the mis-hybridization occurring under the lower stringency conditions employed.

5

Step e):

Preferably step e consists of adding a thermoresistant DNA polymerase to the diluted cDNA solution of step d) and performing a further set of amplification reaction cycles without adding further primers.

10

Following dilution of DNA the amplification of step e) is performed without adding any primers to the diluted cDNA solution obtained at step d). Because no exogenous primers are added, the annealing step results in the hybridization between different amplified DNA molecules initially present in the diluted cDNA solution, which are then elongated before the resulting duplex elongated cDNA molecules are denatured at the end of each amplification cycle.

15

Without wishing to be bound by any particular theory, it appears that the "self priming" amplification of step e) also results in an increase of the number of bridged DNA molecules having a high molecular weight and therefore in an increase in the number of bridged but appropriately amplified genes from the sample.

20

Preferably, the amplification reaction of step e) is performed for a number of amplification cycles ranging from 20 to 40 amplification cycles, more preferably from 25 to 35 amplification cycles and is most preferably of about 30 amplification cycles. However, other cycle numbers could be envisaged. One parameter of the optimum number of cycles required is determined by the polymerase used.

25

Preferably, the amplification reaction of step e) is performed at hybridization conditions of low stringency, but with a greater stringency than the hybridization conditions used in the amplification reaction of step c). Typically, the magnesium concentration generally used is up to 4,5mM, preferably between 1.5 mM and 4.5 mM magnesium and most preferably about 3.5 mM. In these amplification conditions, each amplification cycle comprises the following steps of:

30

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 85°C and 95°C;

(ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature comprised between 55°C and 75°C;

5 (iii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature comprised between 65°C and 75°C;

(iv) reiterating steps (i) through (iii) for the desired number of cycles.

10 In a most preferred embodiment, the amplification reaction of step e) comprises a denaturation step at 92°C, an annealing step at a temperature comprised between 55°C and 72°C, for example 55°C, 60°C, 65°C or 72°C, and an elongation step at 72°C in the presence of a suitable DNA polymerase.

15

Step e'):

A preferred embodiment of step e') comprise a combination of the following steps f) and g).

20 Step f):

The amplification mixture which contains a population of amplified heterogeneous cDNA molecules is then submitted to a further step (step f) wherein the high molecular weight cDNA species, preferably
25 those having a length of at least 4.5 kb, are separated.

Step g):

In a preferred embodiment step g) consists of confirming the presence of at least one nucleic acid sequence species contained in the high
30 molecular weight cDNA separated at step f)

The high molecular weight cDNA species previously separated at step f) can readily be used, typically for detecting the presence of at least one nucleic acid sequence of interest.

Alternatively, the amplification method may comprise an
35 additional amplification step following step f), which consists of

submitting at least a part of the high molecular weight DNA molecules separated at step f) to a further amplification reaction using a pair of primers, wherein a first primer comprises a portion of the first heel sequence and the second primer comprises a portion of the second heel sequence.

Step g) of the amplification method comprises anyone of the following methods:

- (i) detection of the sequences of interest with specific oligonucleotide probes;
- (ii) amplification of the sequences of interest with specific oligonucleotide primers;
- (iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or
- (iv) *in vitro* RNA transcription, either for hybridization assays or for further reverse transcription optionally using unlabelled or labeled substrate followed by gene specific PCR or hybridization.

In part (iv) of step g), the resulting cDNA may also be submitted to *in vitro* transcription. In this context, it is essential that one of the primers comprises a RNA polymerase binding site such as the T7 RNA polymerase promoter. The RNA generated can then be subjected to further process steps, for instance either by being labeled and attached to DNA arrays for hybridization experiments or by being reverse transcribed, optionally using a fluorescent, radioactive or otherwise labeled substrate, to generate labeled cDNA strands. The resulting labeled cDNA can then be hybridized to a DNA array or used in gene-specific PCR experiments.

It is to be noted that the labeling of any of the reactants used in any one of the 3 embodiments of the invention, although optional, can be very useful in that it allows the skilled person to directly hybridize to a DNA array the products of the process of the present invention.

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer;

5 d') cutting any large DNA molecules and preventing bridge formation in subsequent steps by suppressing the heel portions of at least one said first or second heeled primer

e') increasing the amount of long double strand products with sequences more 5' from the original mRNA.

10

Preferably, the synthesis of the first and second cDNA strands in steps a) and b) are performed under the same conditions defined for steps a) and b) of the first embodiment.

15 Step c):

Typically, the first amplification reaction of step c) of this second method is performed under low stringency hybridization conditions.

The low stringency hybridization conditions used at step c) increase the chances to elongate any sequence present initially in the sample containing the first and second cDNA strands population.

20 Preferably, the amplification reaction of step c) includes the following steps of:

(i) obtaining single stranded DNA molecules at a temperature comprised between 85°C and 95°C;

25 (ii) annealing the primers to the single stranded DNA molecules at a temperature comprised between 45°C and 65°C;

(iii) elongating the annealed DNA molecules at a temperature comprised between 65°C and 75°C, preferably between 70°C and 75°C in the presence of a concentration of 4.5mM magnesium;

30 (iv) reiterating steps (i) to (iii) for the desired number of cycles.

In a most preferred embodiment, the amplification reaction of step c) includes a denaturation step at 92°C, an annealing step at 60°C and an elongation step at 72°C.

In another preferred embodiment, the amplification reaction step of the first and second cDNA strands comprises between 30 and 50

35

amplification cycles, more preferably between 35 and 45 amplification cycles, most preferably about 40 amplification cycles. However, other cycle numbers could be envisaged. One parameter of the optimum number of cycles required is determined by the polymerase used.

5

Step d'):

In a preferred embodiment of step d') consists of incubating the product obtained at step c with at least one restriction enzyme that specifically recognise the cleavage site in particular a rare restriction sites included in the primers.

10

Incubating the cDNA strands obtained at the end of the first amplification reaction step c) as shown in embodiment I with the corresponding cleavage agent such as a restriction endonuclease results in the cleavage of the high molecular weight cDNAs produced at this step. This prevents an increase in the number of the high molecular weight cDNA species that would have been generated during the second amplification reaction step e) (of embodiment 1). This step also serves to remove DNA sequences which can compete with the gene specific primers used later on in gene specific PCR. Furthermore, cutting and removing the resulting products increases the efficiency of *in vitro* RNA transcription from amplified DNA.

15

20

According to a specific variant of the second embodiment, the DNA molecules amplified in step c) are incubated in step d) with two restriction enzymes recognizing the rare cleavage site in particular a rare restriction sites of the first and the second heeled primers.

25

As a preferred variant, the rare cleavage site in particular a rare restriction site sequence present at the 3' end of the heel of at least the second heeled primer is a sequence recognized by the MluI restriction enzyme.

30

Restriction cleavage in step d) is performed using the conventional restriction cleavage techniques well known to those skilled in the art such as described for example in Sambrook, J., Fritsch, E.F. , and T. Maniatis, (1989, Molecular cloning: A laboratory Manual . 2nd ed.; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

35

Step e'):

In a preferred embodiment step e') is a combination of the following steps e) and f).

5 Step e):

The cleavage step can be followed by step e) wherein the product of step d) is diluted by an order of magnitude of 2 to 100 times in order to almost completely eliminate the primers used for the first amplification of step d). This favours the phenomenon of self priming (10 as shown in figure 3) in the further set of amplification reaction cycles of step f).

As shown in Figure 3, in the absence of added primers, and after strand separation at 92°C, short strands (e.g. strand B) will be able to serve as primers on complementary longer strands (e.g. strand A),15 resulting in an increase in the amount of double stranded gene specific sequence 5' to the reverse transcription primer site. Note that removal of the second strand primer heel, facilitates this process since the heel primer sequence is not complementary to the gene sequence of strand A. Thick bars on the right side of the diagram represent the reverse20 transcription primer heel, while thick bars on the left represent the second strand cDNA primer heel.

More preferably, the product of step d) is diluted 10 to 80 times and is most preferably diluted about 40 times.

25 Step f):

In a preferred embodiment step f) consists of adding a thermoresistant DNA polymerase to the diluted sample of step e) and performing a further set of amplification cycles without adding further nucleic acid primer.

30 Subsequently to the dilutions of step e), a further set of amplification cycles without adding further nucleic acid primers can advantageously be performed in a step f).

In a preferred embodiment, each amplification cycle of step f) comprises the following steps of:

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 85°C and 95°C;

(ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature comprised between 55°C and 75°C;

5 (iii) elongating the annealed DNA molecules using a thermo-resistant DNA polymerase at a temperature comprised between 65°C and 75°C;

(iv) reiterating steps (i) through (iii) for the desired number of cycles.

10

In a preferred variant of this embodiment, the denaturation step is performed at 92°C, the annealing step is performed at 55°C, 60°C, 65°C or 72°C and the elongation step is performed at 72°C.

15 In another preferred variant, the amplification cycles carried out in step f) compris between 10 and 40 cycles, more preferably between 25 and 35 cycles and most preferably about 30 cycles. However, other cycle numbers could be envisaged. One parameter of the optimum number of cycles required is determined by the polymerase used.

20 The set of amplification cycles carried out in step f) is preferably performed under low stringency hybridization conditions, the presence of about 3.5 mM magnesium.

25 In a specific variant of the second embodiment, the method comprises a further step wherein the DNA molecules obtained at step f) having a length of less than 50 base pairs are discarded from the reaction mixture.

Step g):

30 Furthermore, step g) can comprises one or several of the following methods:

(i) detection of sequences of interest with specific oligonucleotide probes;

(ii) amplification of sequences of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or

(iv) *in vitro* RNA transcription, either for hybridization assays or for further reverse transcription using unlabeled or labeled substrate
5 followed by gene specific PCR or hybridization.

It is important to note that the amplified cDNA obtained from the reverse transcription and amplification of the nucleic acid sequences of the sample may be submitted to *in vitro* transcription either immediately
10 after step d) if the appropriate concentration of cDNA is present in the sample or after further amplification such as through steps e), f) and g). In this context, it is essential that at least one of the primers comprises a RNA polymerase binding site such as the T7 RNA polymerase promoter. The RNA generated can then be subjected to further process steps, for
15 instance either by being labeled and hybridized to DNA arrays or by being reverse transcribed, optionally using a fluorescent, radioactive or otherwise labeled substrate, to generate labeled cDNA strands. The resulting labeled cDNA can then be hybridized to a DNA array or used in gene-specific PCR experiments.

20

It is to be noted that the labeling of any of the reactants used in the above method, although optional, can be very useful in that it allows the skilled person to directly hybridize on a DNA array the products of the process of the present invention.

25

PRIMERS

It is to be noted that although the presence of a cleavage site is an important feature of the second embodiment, this cleavage site can be located either on the first heeled primer, on the second heeled primer,
30 on both primers or on primers used in step c). However, it is necessary that at least one primer comprise a cleavage site.

A) First heeled primers

For performing this second embodiment of the method, the first heeled primer population consists of a population of nucleic acids comprising, from 5' end to 3' end:

- (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a) and wherein the heel sequence optionally includes the nucleotide sequence of a rare cleavage site in particular a rare restriction site located at its 3' end;
- (ii) An optional but preferably present RNA polymerase promoter sequence,
- (iii) an oligo dT sequence of 15 to 25 nucleotides in length;
- (iv) a variable sequence of 2-4 nucleotides in length. This sequence is able to hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof, wherein substantially every possible variable sequence combination is found in said first heeled primer population.

Typically, the variable sequence of 2-4 nucleotides in length of the first heeled primer is selected among the following variable nucleotide sequence: 5'-(A or G or C)-N₁₋₃-3', wherein N is a nucleotide selected from A, T, C or G.

In a specific embodiment of this second amplification method, the first heeled primer may therefore also include the sequence of a rare cleavage site in particular a rare restriction site. **cleavage site in particular a rare restriction site cleavage site in particular a rare restriction site cleavage site in particular a rare restriction site**

The sequence of a rare cleavage site in particular a rare restriction site is usually located within or close to the 3' end of its heel sequence. In the context of the present invention, 'close to the 3' end' is intended to designate that the cleavage site in particular a rare restriction site is to be positioned so as to leave as few bases as possible from the heel after restriction enzyme cutting so as to avoid subsequent aberrant hybridization between the remaining and generated sequences.

Preferably, the cleavage site in particular a rare restriction site is selected from the so-called 'rare cutter' the group which comprises, for example, Not1 BssHII, NarI, MluI, NruI and NaeI.

Preferably, the cleavage site in particular a rare restriction site of the first heeled primer is identical to the cleavage site in particular a rare restriction site of the second heeled primer.

Alternatively, the cleavage site in particular a rare restriction site of said first heeled primer may be different from the cleavage site in particular a rare restriction site of the second heeled primer.

B) Second heeled primer

The second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

- (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a) wherein the heel optionally sequence includes the nucleotide sequence of a rare cleavage site, in particular a rarely used site, located at its 3' end;
- (ii) An optional but preferably present RNA polymerase promoter sequence,
- (iii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of 4 to 7 nucleotides is found in said second heeled primer population; and
- (iv) a second variable nucleotide sequence that was calculated to hybridize on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

Preferably, the cleavage site is located within the heel sequence. More preferably, the cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence of the second heeled primer population, and is a rarely occurring cleavage site in particular a rare restriction site in the genome from which the initial mRNAs are expressed.

Most preferably, the cleavage site in particular a rare restriction site is selected among the cleavage site in particular a rare restriction sites that are found less than once every 20 kb in the genome of the organism from which the cDNA amplification is sought.

In mammals, and more particularly in the rat, such rare cleavage site in particular a rare restriction site is selected from the so-called 'rare cutter' group of cleavage site in particular a rare restriction sites which comprises, for example, Not1, BsshII, NarI, MluI, NruI and NaeI.

Preferably, the heel sequence of the second heeled primer consists of the nucleotide sequence of SEQ ID N°2, CTGCATCTATCTAGTACGCGT.

In a preferred embodiment of the second heeled sequence, said second variable sequence is chosen from the group of sequences consisting of 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3', such that each of said second variable sequence is found in said second heeled primer population.

15

KITS

The invention further relates to a kits for the amplification of the mRNA species present in a sample, wherein said kit compris:

- (i) a first heeled primer population; and
- (ii) a second heeled primer population, as defined above for either embodiments I or II.

The invention also pertains to a kit for the amplification of the mRNA species presenting a sample wherein said kit further comprises:

- (iii) a first primer consisting of the heel sequence of the first heeled primer;
- (iv) a second primer consisting of the heel sequence of the second heeled primer.

In a specific embodiment, the mRNA amplification kit may further comprise one or more restriction enzymes that recognize the rare cleavage site in particular a rare restriction site sequence that may be present in the heel sequence of the heeled primers.

In another preferred embodiment, said mRNA amplification kit may further comprise a restriction enzyme that recognize the rare cleavage site in particular a rare restriction site sequence that may be present in the heel sequence of the first heeled primer.

In yet another preferred embodiment, the kit may also include a suitable RNA polymerase.

THIRD EMBODIMENT OF THE INVENTION

5

According to this third amplification method of the invention, higher stringency hybridization conditions are used to prevent the generation of bridged nucleic acids. In the second embodiment, bridges were cleaved by using a primers containing a rare cleavage site in its heel sequence. This allowed cleavage by a cleaving agent, preferably a restriction endonuclease, of the long cDNA molecules formed during the first set of amplification cycles.

The conditions for performing the third embodiment have been chosen to further reduce bridge formation. Such conditions include for example, (apart from the optional presence of a restriction site on the primer) increasing the stringency of hybridization with respect to the stringency used in embodiment 1 or 2, for example by optimizing buffer conditions which will in turn decrease mis-hybridizations and/or increasing the GC content of the primers which allows elevated annealing temperatures, which also reduces mis-hybridization and increases the distance between hybridized paired oligonucleotides.

These higher stringency hybridization conditions may be met according to two alternatives of this third embodiment which are described hereunder.

The method of embodiment III is a method to increase the number of nucleotide sequences corresponding to an mRNA species present in a sample in a low quantity comprising the steps of:

- a) reverse transcribing the mRNA species using first heeled primer population to provide first strand cDNA species;
- b) synthesizing second cDNA strands using a second heeled primer population;
- c) amplifying said second cDNA strands resulting from step b) over a number of amplification cycles in order to generate second cDNA

strands comprising heels at both ends and increasing the number of second cDNA strands corresponding to long mRNA species present initially in the sample to be assayed;

5 d) amplifying the DNA molecules resulting from step c) under hybridization conditions which are of a higher stringency than those of step c) and which enable reduction of the synthesis of high molecular weight cDNA molecules; and

e) recovering the population of DNA molecules obtained at step d).

10

PROCESS

Step a)

Preferably, step a) is the same as for the first and the second embodiments, except that the first heeled primer population comprises a heel sequence that must have a GC content ranging from 60% to 80% and which is most preferably of about 75%, in order to permit an increase in the stringency of the hybridization conditions used in the first set of amplification cycles of step c), thereby reducing the formation of nucleic acid bridges inside the amplified cDNA molecule.

20

Step b)

Step b) of synthesis of the second cDNA strands is also performed at hybridization conditions of a higher stringency than the hybridization conditions used in step b) as described for the first and the second embodiments of the invention.

25 Step b) is preferably performed at high stringency conditions. A preferred example of high stringency conditions is as follows: synthesizing second cDNA strands using a second heeled primer population preferably at a concentration ranging between 0.02 to 200 ng per reaction in the following conditions;

- 30 (i) adding the primers to the cDNA product obtained at step a);
(ii) obtaining single stranded DNA molecules at a temperature comprised between 85°C and 95°C preferably for a period of time which ranges from 2 to 5 min,;

(iii) adding a thermoresistant DNA polymerase and optionally a thermoresistant proof reading enzyme to the mixture obtained at step (ii);

(iv) Optionally maintaining the temperature of the mixture at approximately 94°C during a period of time up to 5 min

5 (v) annealing the healed primers to said single stranded DNA at a temperature comprised between 40°C and 72 °C;

(vi) elongating the annealed DNA molecules at a temperature comprised between 60°C and 75 °C;

10 High stringency hybridization conditions are notably obtained according to the specific structural features of the second healed primer used.

Preferably, step b) is performed in the presence of a magnesium concentration generally up to 5 mM magnesium, preferably between 3 and 5mM magnesium, most preferably of 3.5 mM.

15 The thermoresistant DNA polymerase is preferably added at step (b) in an amount that ranges from 3U to 5U, most preferably 4.5U DNA polymerase in a volume of 1µl. Optionally step (b) is performed in the presence of both a thermoresistant DNA polymerase and a proof reading enzyme.

20 This enzyme being added at the same time as the DNA polymerase and in an amount which preferably ranges from 0.1 U to 0.5U, most preferably 0.25 U and is admixed with the DNA polymerase in a volume of 1µl.

25 With regard to step (b) (iv), it is performed for a period of time preferably from 1 min to 3 min, most preferably during 2 min.

With regard to step (b) (v), it is preferably performed at a temperature of 50°C for a period of time generally up to 10 min, preferably between 4 min and 10 min, more preferably 6 min and 8 min and most preferably 7.5 min.

30 With regard to, step (b) (vi), it is preferably performed at a temperature of 72°C for a period of time comprised between 1 min and 5 min, preferably between 2 min and 4 min and most preferably during 2.5 min.

35 High amounts of second healed primer population used in steps b) and c) increases the probability of annealing of at least one primer to

every sequence contained in the first cDNA strands previously synthesized at step a).

Step c)

5 Although the inventors do not wish to be bound to any particular theory, it appears that through the successive cycles of the amplification reaction of step c), the sequences that contain at their 5'-end the heel sequence of the second heeled primer will anneal to the first strand cDNA in order to generate second cDNA strands comprising heels at
10 both ends. These repetitive cycles of step c) increase the chances of detecting every first strand present in the reaction mixture of step b).

As a consequence, complementary sequences to the 5' end of the gene sequences present in the sample are generated.

15 In a first preferred embodiment of step c), no denaturation is performed during the successive cycles. This situation permits an increased efficiency in long sequence elongation by allowing the polymerase to work through several cycles without removing the primers or short DNA sequences hybridized to the first strand. Furthermore, the
20 inventors believe that the polymerase may actually displace small sequences hybridized to the first strand during the elongation to favor the extension of longer sequences already hybridized to this first strand.

 In a second preferred embodiment of step c), denaturation is performed under mild temperature conditions, preferably in the range of
25 80 to 85°C. In these conditions, small mismatched sequences, generally of less than 50 bp in length and preferably at least the second heel primers, are removed from their hybridization site on the first strand and are thus available for further priming in subsequent reactions. The further increases the yield in the amplification of the second strand cDNA.

30 In a third preferred embodiment of step c), denaturation is performed under usual temperature conditions, preferably in the range of 85 to 95°C.

 The first and second cDNA strands previously synthesized are
35 preferably amplified over a number of amplification cycles with the

second heeled primer at a concentration ranging between 0.02 to 200 ng per reaction, preferably 0.02 to 100 ng, more preferably between 1 and 50 ng and most preferably between 1 and 10 ng.

The preferred amount of second heeled primer population used
5 in step c) increases the probability of annealing of at least one primer to every sequence contained in the first and second cDNA strands previously synthesized at steps a) and b).

Preferably, a population of approximately 4^{17} primers is used
10 during the amplification reaction of step c). This increases the chances of each gene sequence annealing to at least one primer.

The availability of each primer is increased by multiplying the number of cycles in the amplification reaction of step c). Preferably, step (c) is performed in the presence of 4.5 mM magnesium between 30 and 50 amplification cycles, more preferably between 35 and 45 amplification
15 cycles and most preferably about 40 amplification cycles.

Advantageously, the amplification reaction of step c) is performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme.

The presence of a thermoresistant proof reading enzyme in the
20 amplification buffer allows a significant increase in the quality of the sequences that are synthesized during the elongation step of each amplification reaction cycle.

Most preferably, step c) comprises a step wherein the heeled primers are elongated in the presence of the DNA polymerase and
25 optionally the proof reading enzyme at a temperature ranging between 40 and 72°C.

Optionally, an annealing step may be performed between the denaturation step and the elongation step, at 40°C, a temperature wherein the DNA polymerase is almost prevented to synthesize DNA.

30 Preferably, step c) comprises an elongation step wherein the annealed DNA molecules are elongated at a temperature comprised between 65 and 75°C in the presence of a thermoresistant DNA polymerase.

In a preferred alternative variant, step c) can comprise
35 the step of amplifying second cDNA strands resulting from step b) over a

number of amplification cycles with said second heeled primer preferably at a concentration ranging between 0.02 to 200 ng per reaction in the following conditions;

5 (i) optionally obtaining single stranded DNA molecules at a temperature comprised between 80°C and 95°C, in the presence of a thermoresistant DNA polymerase,

(ii) annealing the second strand primers to the first strand (single stranded) DNA molecules at a temperature comprised between 40°C and 72°C; preferably between 40°C and 60°C,

10 (iii) elongating the annealed DNA molecules at a temperature comprised between 65°C and 75°C optionally in the presence of a thermoresistant DNA polymerase;

(iv) repeating steps (ii) and (iii) (with (i) as an option) for the desired number of cycles.

15 Preferably, steps (c) (ii) to (iv) are repeated for 10 to 60 cycles, preferably from 20 to 50 cycles and most preferably about 20 or about 40 cycles.

In a preferred variant of step (c), a population of second heeled primers is added at step (b).

20 Preferably, step c) is performed in the presence of a magnesium concentration up to 5 mM and most preferably of 3.5 mM.

In a most preferred variant, step (c) is performed in the presence of both a thermoresistant DNA polymerase and a proof reading enzyme.

25 Advantageously, the proof reading enzyme is added at the same time as the DNA polymerase and in an amount which ranges from 0.1 U to 0.5U, most preferably 0.25 U and is admixed with the DNA polymerase in the volume of 1µl.

30

Step d)

The third embodiment further comprises a second set of amplification cycles which are performed at step d) (referred to above) under more stringent hybridization conditions. This serves to amplify all
35 the cDNAs bearing heel sequences with minimum bridge formation and,

hybridization conditions specified, and (3) a mixture of the primers (1) and (2), wherein the total concentration of primers preferably ranges between 0.02 and 500 ng per reaction in the following conditions:

- (i) adding the primers to the cDNA product obtained at step c);
- 5 (ii) obtaining single stranded DNA molecules at a temperature comprised between 80°C and 95°C;
- (iii) adding a thermoresistant DNA polymerase;
- (iv) maintaining the temperature at a range from 80°C to 95°C for a period of time preferably comprised between 5 sec to 15 min;
- 10 (v) annealing the primers to the said single stranded DNA and elongating the annealed DNA molecules at a temperature comprised between 65°C and 75 °C;
- (vi) carrying out steps (iv) and (v) for a desired number of cycles.

15

With regard to step d), in a preferred embodiment, step d) (iv) is performed at a temperature of 94°C. According to these conditions, the reaction mixture contains essentially the single stranded cDNA products obtained at step c), the amplification primers as well as the thermoresistant DNA polymerase which is not active at this high temperature.

20 Preferably, at the first occurrence of step d) (iv) in the amplification method, the temperature is maintained in the range from 80°C to 95°C, most preferably 94°C, for a period of time up to 3 minutes, most preferably 2 minutes. For the further occurrences of step d) (iv), then the temperature ranges from 80°C to 95°C, most preferably 94°C, and is maintained up to 60 sec, most preferably 20 sec.

25 At step d) (v) the primers are annealed to the single stranded DNA molecules at a temperature wherein the thermoresistant DNA polymerase is able to elongate the primers using the cDNA molecules as templates.

30 Preferably, step d) (v) is performed at a temperature comprised between 68°C and 74°C, most preferably 72°C.

In a preferred embodiment, step d) (v) is performed during a period of time comprised between 1 min and 10 min, most preferably 5 min.

5 In a preferred embodiment, the last occurrence of step d) (v),, is performed during a period of time comprised between 10 and 60 min, preferably between 25 and 40 min, most preferably during 35 min.

Step d), (vi) preferably comprises between 10 and 50 amplification cycles.

10 Preferably, the amplification reaction of step d) is performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme, also step d) is preferably performed in the presence of a concentration of magnesium comprised between 2 and 5 mM.

15 In a preferred expression of the second alternative of the third embodiment, the respective concentration of primers at step (d) ranges from 0.02 to 500 ng.

20 Step d) which includes steps (i) to (vi) as described above, is preferably performed when the initial sample contains a large amount of mRNA, such as for example an amount of mRNA corresponding to the whole mRNA that can be found after extraction from about 100 cells (e.g. 100 mammalian cells).

25 In this situation step d) is preferably performed in the presence of a concentration of magnesium up to 3 mM, most preferably 2 mM.

30 If the initial sample contained a small amount of mRNA, such as the amount of mRNA that may be found after extraction from 1 to 10 cells, step d) above will preferably comprise further steps of amplifying the products obtained at step d) (vi) of the second alternative.

In this situation, step d) further comprises the steps of amplifying the DNA molecules obtained at step d) (vi) over a number of amplification cycles with primers selected from the group consisting of
35 (1) a primer comprising a portion of the heel sequence of the first heeled

primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, (2) a primer comprising a portion of the heel sequence of the second heeled primer which portion is of a nucleotide length sufficient to
 5 hybridize with its complementary sequence under the hybridization conditions specified, and (3) a mixture of the primers (1) and (2), wherein the total concentration of primers preferably ranges between 0.02 and 200 ng per reaction in the following conditions:

(vii) obtaining single stranded DNA molecule at a temperature
 10 comprised between 80°C and 95°C;

(viii) adding a thermoresistant DNA polymerase to the single stranded DNA molecules obtained at step (vii);

(ix) annealing and elongating the single stranded DNA molecules at a temperature comprised between 65°C and 75°C;

15 (x) carrying out steps (vii) and (ix) for a desired number of cycles.

With regard to the magnesium concentration used at step (d), these are preferably of (a) 2.5 mM magnesium at steps (d) (i) to (vi) and (b) 2mM magnesium at steps (d) (vii) to (x).

20 As for amplification reaction steps (d) (i) to (vi) these are performed with a respective concentration of primers which ranges from 0.02 to 90 ng, preferably from 10 to 50 ng, most preferably about 30 ng, then step (d) (vii) to (x) is performed with a respective concentration of primers that ranges from 50 ng to 300 ng, preferably from 65 ng to 200
 25 ng and most preferably about 100 ng.

Thus, when the initial sample contained a small amount of mRNA species, step (d) (i) to (x) are preferably performed, using a total amount of primers ranging from 0.02 to 500 ng, preferably from 60 to 300 ng and most preferably about 130 ng.

30 Preferably, step (d) (x) of the second alternative of the third amplification method described above comprises between 20 and 60 amplification cycles, preferably between 30 and 50 amplification cycles and most preferably about 40 amplification cycles.

35 As it is detailed above, when step (d) is performed by carrying out steps (i) to (x), the first set of amplification reactions of steps (i) to (vi)

is performed with a smaller amount of primers than when step (d) is performed by carrying out solely steps (i) to (vi). This lower amount of primers added at step (i) in this specific situation will permit a reduction in the level of mis-hybridizations in the first set of amplification reactions.

5 Thus the products obtained at step d) (vi) are fully representative of the mRNA species initially contained in the sample. According to this variant of the embodiment, the second set of amplification reactions, namely steps (d) (vii) to (x) will increase the amount of material initially amplified at steps (d) (i) to (vi).

10 According to the method above, the amplification reaction steps (d) (viii) to (x) are preferably performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme.

15 Preferably, the amplification reactions steps (d) (viii) to (x) are performed in the presence of a concentration of magnesium up to 4 mM, preferably between 1.6 and 2.5 mM and most preferably at a magnesium concentration of 2.0 mM.

20 In a preferred embodiment, the respective concentration of primers used at steps (d) (vii) to (x) ranges from 10 to 500 ng, and most preferably from 30 to 300 ng.

Step e)

25 With regard to step e) (referred to above) the method may comprise a further step wherein the DNA molecules obtained at step e) having a length of less than 50 bp are discarded from the reaction mixture.

Step e')

30 In an advantageous alternative variant of this embodiment, step d) is followed by the following steps :

i) incubating the DNA molecules obtained at step e) with at least one restriction enzyme that specifically recognizes a restriction site included in the heeled sequence of the first and/or second heeled primer; and/or

Step f) comprises any one of the following methods:

- (i) detection of sequences of interest with specific oligonucleotide probes;
- (ii) amplification of sequences of interest with specific oligonucleotide primers;
- (iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or
- (iv) *in vitro* RNA transcription, either for hybridization assays or for further reverse transcription using unlabeled or labeled substrate followed by gene specific PCR or hybridization.

It is important to note that, the resulting cDNA may be submitted to *in vitro* transcription, either immediately after step c) if the appropriate concentration of cDNA is present in the sample or after further amplification such as through step d) or through optional steps of e') described above. In this context, it is essential that one of the primers comprises a RNA polymerase binding site such as the T7 RNA polymerase promoter. The RNA generated can then be subjected to further process steps, for instance either by being labeled and hybridized to DNA arrays or by being reverse transcribed, optionally using a fluorescent, radioactive or otherwise labeled substrate, to generate labeled cDNA strands. The resulting product can then be hybridized to a DNA array or used in gene-specific PCR experiments. If unlabelled the products can be attached to a microarray base and be hybridized to labeled oligonucleotides.

It is to be noted that the labeling of any of the reactants used in this embodiment of the invention, although optional, can be very useful in that it allows the skilled person to directly attach or hybridize to a DNA array the products of the process of the present invention.

Alternatively, after step e) or e' RNA transcription can be carried out by first optionally removing low molecular weight DNA, including heel primers, to provide a 'cleaner' environment for subsequent RNA

polymerase reactions to take place. This 'cleaning up' also allows the skilled person to change the buffer solution to a buffer that would be more appropriate for subsequent RNA polymerase reactions.

5 It is important to note that the resulting cDNA may be submitted to *in vitro* transcription. It should be noted that inclusion of the RNA polymerase promoter in the primer allows synthesis of complementary RNA, suitable for hybridising to Gene Chips or arrays bearing sense gene specific oligonucleotides, or for subsequent reverse transcription
10 and hybridising of the resultant cDNA to antisense gene specific oligonucleotides. In contrast, inclusion of the RNA polymerase promoter in the second heeled primer allows synthesis of sense RNA, suitable for hybridising to arrays bearing antisense oligonucleotides, or for subsequent reverse transcription and hybridization of the resultant cDNA
15 to GeneChips or arrays bearing sense gene specific oligonucleotides. In this context, it is essential that one of the primers comprises a RNA polymerase binding site such as the T7 RNA polymerase promoter. The RNA generated can then be subjected to further process steps, for instance either by being labeled and hybridized to DNA on arrays or by
20 being reverse transcribed, optionally using a fluorescent, radioactive or otherwise labeled substrate, to generate labeled cDNA strands. The resulting product can then be hybridised to a DNA array, or attached to a support (e.g. glass, nylon, silicon etc) for subsequent hybridisation with other nucleic acids, or used in gene-specific PCR experiments.

25 .

PRIMERS

The specific structural features of the primers used in this embodiment (first and second heeled primers, primers used in step c)
30 include an increase in GC content as compared to primers of embodiments I and II.

Thus, the primers used in this embodiment comprise a heel sequence having a GC content ranging from 60% to 80%, most preferably of about 75%. This increase in the GC content permits an
35 increase in the stringency of the hybridization conditions used in the first

set of amplification cycles of step c), thereby preventing the generation of nucleic acid bridges inside the amplified cDNA molecules and thus preventing the synthesis of the high molecular weight cDNA species observed during step c) of the first and second embodiments.

5

In a preferred embodiment, the above primers comprise at least one cleavage site in their heel sequences or at the 3' end of their heel sequence.

10 In another aspect of the third embodiment of the present invention, the first heeled primer population consists of a population of nucleic acids comprising, from 5' end to 3' end:

- 15 (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the first strand cDNA nor the mRNA molecules initially present in the sample;
- (ii) An option but preferably present RNA polymerase promoter site;
- (iii) an oligo dT sequence of 15 to 35 nucleotides in length; and
- 20 (iv) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof, wherein substantially every possible variable sequence combination is found in said first heeled primer population.

25 According to this specific embodiment of the method, the variable sequence of 2 to 4 nucleotides is selected among the following variable nucleotide sequence : 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from A, T, C or G.

30 Preferably, the first heeled primer includes the sequence of a rare restriction site which may be located at any position within the heel sequence and preferably at the 5' end or at the 3'end of the heel sequence of said first heeled primer.

Preferably, the oligo dT sequence has a length comprised between 20 and 35, more preferably between 25 and 35 and is most preferably of about 30 nucleotides in length.

The first variable sequence of the second heeled primer population has preferably 15 to 20 nucleotides in length and is most preferably of about 17 nucleotides in length. The first variable sequence
 5 of the second heeled primer population is longer the first variable sequence of the second heeled primer used to perform the first and second embodiments described above and are thus suitable to stabilize every second heeled primer of the population to its corresponding complementary DNA sequence during the annealing and the elongation
 10 step of the first and second set of amplifications cycles of steps c) and d).

A longer first variable sequence for stabilizing the primers belonging to the second heeled primer population was required, particularly due to the greater length of the heel sequence, which is
 15 preferably comprised between 25 and 30 nucleotides in length and is most preferably of about 27 nucleotides in length.

In a first preferred embodiment of the second heeled primer population, each nucleic acid sequence also comprises a second variable nucleotide sequence preferably selected according to the criteria
 20 set forth in the first embodiment. Preferably, the second variable sequence of the second heeled primer is chosen from the group of sequences consisting of 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3', such that each of second said variable sequence is found in said second heeled primer population.

25 In a preferred variant, the heel sequence has 28 nucleotides in length.

In a preferred variant of the second heeled primer population for performing the third embodiment, said second heeled primer population comprises a heel sequence of 25 to 30 nucleotides in length, more
 30 preferably about 28 nucleotides in length and having a GC content comprised between 50 and 70%, more preferably between 60 and 70% and is most preferably of about 68%.

In one specific variant of the second alternative of the third embodiment, the heel sequences of the first heeled primer and the second heeled primer are identical.

Alternatively, the heel sequences of the first heeled primer and
5 the second heeled primer share a sequence of at least 15 consecutive nucleotides, preferably at least 20 or 25 consecutive nucleotides.

According to this specific embodiment, the cDNA strands present in the mixture obtained at the end of step (c) of the present method comprise a sequence in their 5' end of at least 15 nucleotides
10 which are complementary to a sequence comprised in their 3' end. In this context, second cDNA strands of a short nucleic acid length that are regenerated during step c) have a high tendency to self-anneal and thus be no longer available for the set(s) of amplification reactions of step (d). Accordingly, the first and second cDNA strands that are amplified when
15 carrying out step (d) of the present method are mainly large cDNA molecules, including cDNA molecules comprising a sequence which is identical or which is complementary to the full length mRNA species initially present in the sample.

20 The heel sequences of the first and second heeled primers preferably comprised the sequence of a rare restriction site located at the 3'-end or at the 5'end of their respective heel sequence, as well as a RNA polymerase binding site, preferably located downstream from the restriction site.

25 In a first variant, the restriction site sequence of the first heeled primer is identical to the restriction site sequence present in the heel of the second heeled primer.

In a second variant, the restriction site sequence of the first heeled primer is different from the restriction site sequence present in
30 the heel of the second heeled primer.

Advantageously, the restriction site sequence included in the heel sequence of the first heeled primer or the second heeled primer is selected from the group of so-called 'rare cutters' which comprises for example Not1, BsshII, NarI, MluI, NruI and NaeI.

35

KITS

The present invention further relates to kits for the amplification of the mRNA species present in a sample, said kit being specifically designed for performing the third cDNA amplification method described
5 above.

Thus, another object of the invention consists of a kit for the amplification of the mRNA species present in a sample, wherein said kit comprises:

- (i) a first heeled primer population; and
 - 10 (ii) a second heeled primer population,
- the first and second heeled primer populations being defined above.

Said amplification kits may further comprises:

- (iii) a first primer consisting of at least a portion of the heel
sequence of the first heeled primer; and
- 15 (iv) a second primer consisting of at least a portion of the heel
sequence of the second heeled primer.

In a specific embodiment of the kit above, the heel sequences of the first heeled primer and of the second heeled primers are identical or alternatively share a common sequence of at least 15, preferably at
20 least 20, most preferably at least 25 consecutive nucleotides in length.

In a specific embodiment, said amplification kit may further comprises a restriction enzyme that recognizes the rare restriction site sequence present in the heeled sequence of the second heeled primer.

In another specific embodiment, said amplification kit may
25 further comprises a restriction enzyme that recognizes the rare restriction site sequence present in the heeled sequence of the first heeled primer.

In yet another embodiment, the kit further comprises a suitable RNA polymerase.

The three mRNA amplification methods of the invention make it
30 possible to amplify large numbers of samples easily and with high sensitivity.

The ability to analyze subsequently the expression of many genes of annealed sequences, both at high and low abundance, in samples taken from as little as a single cell, potentially allows it to be
35 used in high throughput screening systems.

Various kinds of mRNA containing samples may be used as starting materials for performing the cDNA amplification methods of the invention, such as the whole content of a cell cytoplasm or even a portion of the cell cytoplasm such a portion of cytoplasm of neuronal
5 cells and also mRNA molecules extracted from a desired tissue.

cDNA molecules obtained at the end of any one of the three cDNA amplification methods described above can be used for many purposes including:

a) cloning and production of cDNA libraries from small amounts
10 of tissue;

b) sequencing analysis of gene expression in small tissue samples;

c) subtracting the amplified product from two different samples and analysing genes differentially expressed between them such as
15 described by Diatchenko et al. (1996, Proc. Natl. Acad. Sci. USA, vol. 93: 6025-6030), and then by cloning and sequencing the sequences expressed only in one or several tissues;

d) transcribing mRNA using labelled precursors for use on hybridisation arrays, such as described by Duggan , D. J. et al., (1999,
20 Nature Genetics, vol.21: S10-S14);

e) transcribing RNA and reverse transcribing in the presence of labelled precursors for use on hybridising arrays;

f) labelling the cDNA obtained by anyone of the cDNA amplification methods of the invention during the amplification for use on
25 hybridization arrays;

g) diagnosis of aberrant gene expression in small tissue samples from humans, animals and plants.

h) analysis of the effects of drugs and other agents (e.g. infectious agents, carcinogens) on gene expression in vivo and *in vitro*.

30 In the latter case, small tissue samples or cultured cells may be used. In the former case, small tissue samples can be taken from living organisms without any disadvantages since only very small samples are needed;

i) analysis of gene expression in single cells using anyone of
35 the cDNA amplification methods described above.

j) amplification of full length RNA samples from single cells and small samples, for subsequent library making or expression in suitable expression systems.

Preferred embodiments of the invention will now be illustrated, but not limited to, the examples presented hereafter.

EXAMPLE I - Rat brain mRNA amplification using the first embodiment.

mRNA isolated from whole rat brain was reverse transcribed. cDNA derived from 100 pg total RNA (equivalent to the RNA content of between 5 and 10 cells) was amplified according to the first embodiment. After reverse transcription only, gene specific PCR assays were positive when cDNA derived from more than 10 pg of total RNA was used in each assay, as shown in figure 1A. After the first amplification step (c), the majority of the genes were detected using 2.5% of the amplified product in each gene specific assay (i.e. each sample contained material derived from 2.5 pg of the original RNA), with some gene sequences detectable at lower levels, as shown in figure 1B. After step (f) a further increase in sensitivity was observed with all the genes assayed being positive using as little as 0.1% of the amplified product (i.e. amplified cDNA derived from 0.1 pg of the initial total mRNA sample), as shown in figure 1C. Therefore, using this approach, the expression of up to a 1000 genes could be assayed using 0.1% of the final product in each gene specific PCR reaction.

Reverse transcription

Total mRNA was prepared from rat whole brain using the total mRNA isolation system from Promega according to the manufacturer's instructions. Reverse transcription was performed using thermoscript reverse transcriptase or MMLV reverse transcriptase according to the manufacturer's (GIBCO-BRL, Paisley, Scotland) instructions. The reverse transcription primers used were composed of an anchored oligo-dT primer with a specific 5' heel sequence absent from the mammalian data bases. In some instances a RNA polymerase (T7) promoter site

was incorporated at the 3' end of the heel sequence. The primers used were as shown in SEQ ID N°3:

SEQ ID N°3: CTCTCAAGGATCTTACCGCTTTTTTTTTTTTTTTTTT
(A,G,C)(A,G,C,T)

5

Second strand cDNA synthesis was initiated by incubating cDNA derived from 100 pg of total RNA with 25 pg of a mixed primer population consisting of (5'-3'): a 5' heel sequence absent from the mammalian data bases (CTGCATCTATCTAATGCTCC), a stretch of 5 random
10 nucleotides (NNNNN, where N represents A, C, G or T) and a variable pentameric sequence chosen from CGAGA, CGACA, CGTAC and ATGCG, as shown in SEQ ID N°4, 5, 6 and 7. These primers will bind at multiple sites on the first strand cDNA and prime second strand synthesis from such priming sites. After annealing (7.5 mins at 50°C),
15 primer extension was performed for 8 mins at 72°C using ampliTaQ DNA polymerase (0.35 units, Applied Biosystems, Warrington, UK) in PCR-1 buffer containing 67 mM TrisHCl (pH 8.3) 4.5 mM MgCl₂, 6 mM beta-mercaptoethanol, 0.16% bovine serum albumin and 0.5 mM dNTPs.

Subsequently 1 ng (each) of the reverse transcription primer heel
20 and second strand primer heel were added in 5 µl of PCR-1 buffer and the reaction subjected to 10 cycles of 92°C for 0.5 min, 60°C for 1.5 min and 72°C extension of 1 min, followed by a final 10 min extension. A further 10 ng of each heel primer were then added in 20 µl of PCR-1 buffer and subjected to a further 40 cycles (as before). The final product
25 was then diluted to 100 µl with water and samples (2.5 or 5 µl) used for subsequent gene specific PCR assays, or subjected to a further 40 cycles (of 92°C for 0.5 min, 60°C for 1.5 min and 72°C for 1 min, followed by a final 10 min extension) in the absence of added primers. This was performed in a PCR-2 buffer containing 3.5 mM MgCl₂, 45 mM
30 Tris HCl pH 8.8 and, 12.5% sucrose, 0.1 mM cresol red, 12 mM beta-mercaptoethanol, 0.5 mM dNTPs (Pharmacia), with 0.6 U AmpliTaq DNA polymerase (Applied Biosystems). This product was electrophoresed in a 2% agarose gel (E-gel, Invitrogen) and the high molecular weight products isolated from the gel using the Qiagen Gel extraction kit
35 according to the manufacturer's instructions.

Gene specific PCR was performed on samples (2.5 to 10 µl) of amplified cDNA in PCR-2 buffer with gene specific primers at 100 ng/reaction. Following an initial 2 min denaturing step (92°C), each PCR cycle consisted of 0.5 min denaturing (92°C), 1.5 min annealing (55°C), and 1 min elongation (72°C). with a final extension for 10 min at 72°C. The PCR products were then separated by electrophoresis in a 2.5% agarose gel, stained with ethidium bromide and the image recorded. The gene-specific primers used were as follows: α Tubulin, (accession number, V01226, SEQ ID N°8 and 9), β-actin, (accession number, V01217, SEQ ID N°10 and 11), Cyclophilin, (accession number, M25637, SEQ ID N°12 and 13), Adenosine A1 receptor, (accession number, Y12519, SEQ ID N°14 and 15), Adenosine A2A receptor, (accession number, L08102, SEQ ID N°16 and 17), Adenosine A2B receptor (accession number, M91466, SEQ ID N°18 and 19), Adenosine A3 receptor, (accession number, M94152, SEQ ID N°20 and 21), NK1 receptor (accession number, J05097, SEQ ID N°22 and 23), NK2 receptor (accession number, M31838, SEQ ID N°24 and 25), trkA receptor (accession number, M85214, SEQ ID N°26 and 27), trkB receptor (accession number, M55291, SEQ ID N°28 and 29), proenkephalin, (accession number, S49491, SEQ ID N°30 and 31), synaptotagmin 1 (accession number, X52772, SEQ ID N°32 and 33), synaptotagmin 5 (accession number, X84884, SEQ ID N° 34 and 35), mammalian degenerin, (accession number, U53211, SEQ ID N°36 and 37), Glutamate decarboxylase (GAD67, accession number, X57573, SEQ ID N°38 and 39), choline acetyltransferase (not in GenBank/EMBL data bases, see Brice et al., (1989) J. Neursoci. Res., 23, 266-273, SEQ ID N°40 and 41),

Without wishing to be bound by any particular theory, the inventors believe that the increased sensitivity seen after step (e) is due to the removal of products formed during step (c) which compete in the gene specific PCR amplification. These products contain repetitive primer sequences which are capable of priming on the amplified cDNA molecules and thus reduce the efficiency of the gene specific reaction. These products are removed

during step (f), while the amplified gene sequences which have been incorporated into the high molecular weight products during step (e) are retained.

As shown in Figure 1, amplification of cDNA derived from 100
 5 pg of total RNA permits the detection of specific gene sequences by PCR at levels lower than those of unamplified cDNA. (A) dilutions of unamplified cDNA; B) dilutions of the amplified cDNA (step c); C) dilutions of the amplified cDNA (step e). The scale in (A) indicates the amount of total RNA from which the cDNA used in each gene specific
 10 assay was synthesised. In (B) and (C), the scale indicates the amount of total RNA from which the gene specific assay sample was amplified (i.e. 0.1 pg represents one thousandth of the final product obtained after amplification of cDNA derived from 100 pg of total RNA). Gene sequences were detected after amplification (as described in steps (a) to
 15 (c) of the first embodiment of the invention when using amplified product containing as little 1 pg of the initial RNA. After step (e) a further increase in sensitivity can be seen with detection at levels as low as 0.05 pg.

20 **EXAMPLE II: The effect of restriction digestion on the detection of specific sequences after rat brain mRNA amplification using the second and third embodiments.**

25 I. mRNA isolated from whole rat brain was reverse transcribed, and the cDNA derived from 25 pg total mRNA (equivalent to the mRNA content of between 2 and 5 cells) amplified according to Example 1, with (A) or without (B) cutting with Mlu1 as described in step d), followed by steps e) to g), figure 2. Each gene specific assay contained amplified
 30 product derived from 0.6 pg of total RNA. Note the detection of the adenosine A1 and A3 receptor after cutting (A) which were not detected without cutting (B).

II. mRNA isolated from whole rat brain was reverse transcribed, and the cDNA derived from 25 pg total mRNA (equivalent to the mRNA
 35 content of between 2 and 5 cells) amplified according to Example III, with

(A) or without (B) cutting with Mlu1 as described in step g), followed by steps h) to j), figure 2. Each gene specific assay contained amplified product derived from 0.6 pg of total RNA. Note the increased frequency of detection of the low abundance mRNAs (mammalian degenerin (MDEG), A2B receptor) as well as those of medium (GAD67, (glutamate decarboxylase; ChAT, choline acetyltransferase) and high (Synaptotagmin 1) abundance.

cDNA (derived from 25 pg total RNA from rat brain) was prepared and subjected to second strand synthesis as described in Example 1, except the heel of the second strand primers as with SEQ ID N°2, which contains the Mlu1 cleavage site in particular a rare restriction site (ACGCGT) at the 3' end. After amplification to step c) as described in Example 1 (I) or Example 3 (II)), 10 µl of the diluted product was incubated in a total of 20 µl at 37°C for 60 min with 2 units of Mlu1 in 6.0 mM Mg²⁺, according to the manufacturer's instructions (Promega). After addition of EDTA to chelate the Mg²⁺ and incubation at 65°C for 5 mins to inactivate the enzyme, 10 µl aliquots were reamplified in PCR-2 buffer containing 0.625 units of AmpliTaq (and 0.05 units of pfu) DNA polymerases for 40 cycles of 92°C for 0.5 min, 60°C for 1.5 min and 72°C extension of 1 min, followed by a final 10 min extension, in the absence of added primers (I). In (II), 10 µl aliquots were subjected to a further 40 cycles at 92°C for 1.0 min, 95°C for 0.33 min, 72°C for 3 min, followed by a final 15 min. extension. The product was diluted to a final volume of 50 µl or 100 µl and samples subjected to gene specific PCR assays as described in Example 1.

In this second embodiment of the method of the invention, removal of the heel sequence of the second strand primer was designed to increase the sensitivity of the gene specific PCR by cutting of the competitor products described in Example I (so that they no longer compete in any of the subsequent PCR reactions), and also to promote the detection of gene sequences upstream from the reverse transcription primer site. The increased sensitivity due to removal of the primer sequences, is apparent in the increased sensitivity of detection of the gene sequences indicated. It is believed (but the applicants do not wish

The outcome of this procedure was the ability to detect genes by gene specific PCR (as described in Example 1) at dilutions of the amplified cDNA derived from as little as 0.01 pg of total RNA, illustrating that approximately 100,000 gene specific PCR assays could successfully be performed after amplification of cDNA derived from 1ng of total RNA (i.e. the content of approximately 100 cells). In addition product priming/product repair was shown to occur with the detection of a gene sequences 2.4 kb 5' to the reverse transcription priming site (Figure 4).

10

As shown in Figure 4, high stringency amplification cDNA derived from 1000 pg of total RNA as described in embodiment 3 permits increased detection of gene sequences. (I) The scale indicates the amount of total RNA from which the cDNA used in each gene specific assay was synthesised (A), or the amount of RNA from which the gene specific assay sample was amplified (B).

Specific sequences were detected when product amplified from as little as 0.01 pg of the initial RNA was used in the gene specific PCR. (II) Gene specific PCR using cDNA amplified from 2.5 pg of total RNA per gene specific assay. Inclusion of steps g) to i) of Amplification Method 3 also resulted in the detection of NK2 receptor gene sequence located 2350 bp upstream of the polyA splice site (A). This sequence was not detected if step g) was omitted (B). III Amplification of cDNA derived from 1 ng total whole brain RNA by Amplification Method 3 permits the detection of gene specific sequences in 0.006% of the product (equivalent to cDNA amplified from 0.06 pg of total RNA). Control: no amplification.

Furthermore, as shown in (II) of Figure 2, including steps g) to i) in embodiment 3 (A) increases the detection of low abundance messages such as those encoding the adenosine A2B receptor and the mammalian degenerin MDEG, when compared to an amplification which omitted step g) (B). In addition the detection of abundant mRNA species such as that encoding synaptotagmin 1 was also increased.

30

EXAMPLE IV *In vitro* transcription of RNA from cDNA amplified according to embodiments 1 and 3

Incorporation of the T7 promoter into the reverse transcription
 5 primer heel was performed so that RNA could be produced for
 subsequent analysis by hybridisation methods, for instance on oligo
 arrays. The yield of RNA from the amplified cDNA was estimated by
 running two parallel transcriptions, one for RNA synthesis and the other
 containing 35S-UTP as a substrate, so that the incorporation of the
 10 radioactivity into RNA could be used as an index of RNA synthesis.
 After amplification of rat liver cDNA derived from 500 pg of total RNA by
 Amplification Procedure 1 to step (c), *in vitro* transcription resulted in a
 yield of 12.5 micrograms of RNA. Using Embodiment 3 to step (c), the
 yield of RNA from liver cDNA (derived from 500 pg of total RNA) was 34
 15 micrograms (mean of 5 experiments). Similarly the yield from cDNA
 derived from 2500 pg was 90 micrograms (mean of 5 experiments)..
 Inclusion of step g) of Embodiment 3 prior to *in vitro* transcription
 increased the yield of RNA 1.7-fold (mean of two experiments). In order
 to examine the sequence content of the RNA transcribed from cDNA
 20 amplified according to Embodiment 3 (to step c) the RNA was reverse
 transcribed using the heel of the second strand heeled primer. Figure 5
 illustrates that the cDNA derived from the transcribed RNA contained
 abundant gene sequence with actin tubulin and cyclophilin sequences
 being detected in aliquots representing 0.0001% of the RNA so
 25 produced. Therefore it appears that the expression of up to 1,000,000
 genes may be assessed in amplified samples derived from 2500 pg of
 total RNA i.e. RNA derived from approximately 250 cells.

Reverse transcription of liver RNA was performed essentially as
 described in Examples 1 and 3, using primers containing a T7 RNA
 30 polymerase promoter site. The primers used were, SEQ ID N°44,:

CTCTCAAGGATCTTACCGCTAATACGACTCACTATAGGCGCTTTTTT
 TTTTTTTTTTTTTTTTTT
 (A,G,C)(A,G,C,T) and SEQ ID N°45

35

GACTGCCAGACCGCGCGCCTGACGCGTAATACGACTCACTATAGG
GTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

5 (A, C, G) (A, C, G, T)

Subsequent second strand synthesis and amplification was carried out as described in the relevant examples. After step (c) of each Embodiment the amplified cDNA was isolated using a Qiaquick PCR
10 purification kit (Qiagen) according to the manufacturer's instructions to remove primers and other low molecular weight products, and 5 µl aliquots subjected to in vitro RNA transcription using the T7 Megascript kit (Ambion) according to the manufacturer's instructions. After DNase treatment to remove cDNA (DNase 1, 30 min, 37°C), the RNA was
15 isolated using the Rneasy kit (Qiagen). One of the aliquots was transcribed in the presence of 35S-UTP to determine the yield of RNA. The RNA was subsequently ethanol precipitated (75% ethanol, 5% sodium acetate at -20°C for 30 mins), before reverse transcription with MMLV reverse transcriptase according to the manufacturer's instructions.
20 The reverse transcription primers used were part or the whole of the heel sequences of the second strand primers, SEQ ID N°1 and 44

Subsequent gene specific PCR using this cDNA as substrate revealed that as little as 0.0001% of the product could be used in gene
25 specific PCR and result in the detection of gene sequence. The data presented show that embodiment 3 with *in vitro* RNA transcription generates sufficient RNA of good quality for application to cDNA and oligonucleotide arrays. This will permit the analysis of the expression of thousands of genes from tissue samples containing approximately 250
30 cells and with further improvements perhaps from samples as small as single cells.

As shown in Figure 5, *in vitro* transcription of RNA from amplified cDNA contains large amounts of bona fide gene sequence.
35 cDNA derived from 125, 500 and 2500 pg of liver total RNA was

amplified using Amplification Method 3 as far step e) and RNA transcribed. Cutting with the restriction enzyme (step g) was omitted so that the RNA so produced would contain the heel sequence of the second strand primer. This heel primer was then used to prime reverse transcription of the RNA, and the resulting cDNA analysed for the presence of 3 gene sequences. Note that all 3 gene sequences were detected even after 10^6 fold dilution of the product.

Example V. Amplification of rat spinal cord cDNA derived from 1 ng total RNA (equivalent to approximately 100 cells) using the third embodiment.

In example IV it was shown that antisense RNA could be *in vitro* transcribed from cDNA amplified by embodiment 3, this RNA could be applied to gene chips bearing sense probes, or reverse transcribed and applied to microarrays bearing antisense probes. However many microarrays bear sense probes (i.e. they recognise antisense DNA), but are not suitable for the hybridization of labelled RNA samples. In order to maximise the utility of embodiment 3, sense RNA was also transcribed from the amplified cDNA *in vitro*, reverse transcribed and the gene sequence content assessed by gene specific PCR.

Reverse transcription

Reverse transcription (RT) was performed in 10 µl containing; 1x first-strand buffer, 200 Units MMLV reverse transcriptase (BRL), and 0.5 ng first strand primer for 60 min at 37°C. The first strand primer (SEQ ID N°46), ACTGCCAGACCGCGCGCCTGAACGCG TAATACGACTCACTATAGGGTTTTTTTTTTTTTTTTTTTTT(A, C, G) (A, C, G, T), contained (5' to 3') a 26 base sequence absent from the mammalian data bases capable of hybridising to its complement at 72°C in the presence of 2mM Mg^{2+} with an MluI site at its 3' end, the T7 RNA polymerase promoter sequence, and an anchored stretch of oligodTs for hybridising to the 5' end of the polyA sequence of mRNA.

Second strand synthesis

Second strand synthesis was performed by adding an excess of second strand primer (1 ng) (to increase chances of annealing to every first strand sequence) in 4 microlitres of buffer giving a final Mg^{2+} concentration of 3.5mM.

After heating to 80°C, 5 units of Taq (Applied Biosystems, Warrington, UK) was added with 0.25 units of the proofreading enzyme pfu (Stratagene). Adding the Taq at high temperature ("hot start") prevents the enzyme copying mishybridised sequences in the mixture, such mishybridization tending to occur at the low temperatures encountered when setting up the reaction.

Primer annealing occurred at 50°C (7.5 mins decreasing by 10 secs per cycle) and extension at 72°C for 2.5mins. The temperature was cycled between 50°C and 72°C 40 times.

Although not wishing to be bound by theory, is the inventors believe that under these conditions each first strand cDNA will be annealed in multiple positions by the second strand primer. Each cycle permits further annealing by the primer. However, unlike normal PCR, the second strands are not dissociated from the first strand by melting in each cycle, consequently each primer has an equal chance of being extended to the 5' end of the first strand (which bears one of the heels), thus increasing the efficiency of subsequent PCR. It is envisaged that extension of primers at the 3' end of the first strand will displace those nearer the 5' end producing multiple copies of each second strand. The second strand primer contained (from 5' to 3'): a sequence absent from the mammalian data bases which is capable of hybridising to its complement and 72oC in the presence of 2mM Mg^{2+} and standard PCR buffers, an MluI site (ACGCG), the T3 RNA polymerase promoter and a random sequence of 15 bases, SEQ ID N°47:

AAAAC TGCCAGACCGCGCGCCTGAACGCGTCGTATTAACCCTCACT
AAAGGGN15

Amplification reactions

Subsequent PCR was performed by adding 4 microliters in AmpliTaq buffer (Applied Biosystems, Warrington, UK) containing 1.25

mM dNTPs and 33ng of primers (the sequence absent from the mammalian data bases which is capable of hybridising to its complement and 72°C in the presence of 2mM Mg²⁺ with an MluI site) to give a final Mg²⁺ concentration of 2.6 mM.

5 In this example, this primer was common to both the first and second strand primers). After heating to 80°C, 5 units of Taq (Applied Biosystems, Warrington, UK) was added with 0.25 unit of the proofreading enzyme pfu (Stratagene).

The reaction was then subjected to 20 cycles of denaturation
10 (94°C, 20 secs), and annealing with extension (72°C, 5mins). 19 microlitres of AmpliTaq buffer were then added (at 80°C) containing and 0.2 mM dNTPS, 100 ng of primers and giving a final Mg²⁺ concentration of 2.1 mM. 5 units of Taq (Applied Biosystems, Warrington, UK) was then added with 0.25 units of the proofreading enzyme pfu (Stratagene).
15 The reaction was then cycled 40 times as described above, with a final extension at 72°C for 30 min.

After amplification small MW primers and products were removed by passage through a Qiaquick PCR purification kit (Qiagen). The amplified cDNA was then cut with MluI and recleaned using the same kit
20 same prior to subsequent gene specific PCR assays or in vitro transcription. Gene specific PCR was performed as previously described. In vitro transcription of RNA was performed using the Ambion Megascript Kit according to the manufacturers instructions.

After DNase treatment, some of the resulting RNA was reverse
25 transcribed for gene specific PCR. Figure 6 shows the size distribution of the RNA produced from both the T3 and T7 RNA polymerase promoters (i.e. most between 200 and 600 bp with detectable higher molecular weight material). 10% of the product obtained after amplification of cDNA derived from 1 ng of total RNA was in vitro
30 transcribed with T7 polymerase or T3 polymerase and 30% of each RNA applied to a gel. The estimated yields from the two RNA polymerases were 0.5 and 1.5 micrograms respectively. Figure 7A shows that the amplified cDNA contained both rare (A2A receptor) and abundant (e.g. tubulin) gene sequences detectable by gene specific PCR. I:
35 amplification with a second strand primer lacking the T3 promoter, II

amplification with a second strand primer bearing the T3 promoter. Samples were diluted up to 1/3,000 prior to gene specific PCR. Figure 7B shows that the in vitro transcribed sense RNA generated using the T3 RNA polymerase (after reverse transcription to cDNA) also contains
5 abundant gene sequence.

Example VI. Single cell expression analysis using microarrays after cDNA amplification of striatal cholinergic neuron al mRNA using embodiment 3.

10

In order to assess single cell gene expression, mRNA was amplified by the third embodiment using the primers and conditions described in Example V. T3 RNA polymerase was used to generate sense RNA which was then reverse transcribed using fluorescently
15 labelled dCTP (Cy3 or Cy5) for application to glass microarrays bearing sense DNA probes

Harvesting of single cell mRNA

Striatal cholinergic neurons were identified on the basis of their
20 size and electrophysiological characteristics in 300 µm coronal slices from 14-28 day-old male Sprague Dawley rats containing the striatum were viewed with a Zeiss Axioskop microscope (Carl Zeiss Ltd., Welwyn Garden City, U.K.) fitted with a x64 water-immersion objective lens.

Light in the infrared range (>740nm) was used in conjunction with
25 a contrast-enhancing Newwicon camera (Hamamatsu, Hamamatsu City, Japan) to resolve individual neurones within slices (Lee et al., 1998).

The physiological saline bathing the slices contained (mM) 125 NaCl, 25 NaHCO₃, 10 glucose, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂ and was bubbled with 95%/5% O₂/CO₂. The electrode buffer
30 contained 120 K gluconate, 10 NaCl, 2 MgCl₂, 0.5 EGTA, 10 HEPES, 1-4 mM Na₂ATP, 0.3 Na₂GTP, pH adjusted to 7.2 with KOH. 0.5 µg/ml glycogen (Boehringer) and RNase inhibitor (Pharmacia, 0.1 units/µl) were included to facilitate harvesting of RNA from the cells. This buffer also contained 10 fg each of bacterial sequences derived from the trp,
35 thr and lys codons of E. coli. These mRNAs had polyA sequences

attached to the 3' end so that they could be amplified by XTPEA. All solutions were made up in diethylpyrocarbonate (DEPC) treated water. Borosilicate recording electrodes were baked (2h, 250°C) before being pulled to a resistance of between 3 and 5 M Ω . Electrophysiological signals were detected using an Axopatch-1D patch-clamp amplifier (Axon Instruments, CA, USA) and were recorded onto digital audiotape. Following formation of the whole cell configuration, series resistance was partially compensated using the amplifier, and cellular conductance continuously monitored via the injection of hyperpolarising current or voltage. Membrane signals were filtered at 1 kHz and were digitized at 5kHz through a Digidata 1200A/D converter using pClamp 6.0 software (Axon Instruments Inc, CA, USA).

Extraction of Neuronal contents and amplification

The cytoplasm from large cells (>30 μ m in one dimension) was aspirated under visual control into a patch-clamp recording electrode until approximately 40% of the somatic cytoplasm had been collected. Usually the nucleus was sucked onto the end of the electrode until an electrical seal (>0.5G Ω) was formed prior to withdrawal of the electrode to prevent contamination from the slice. Since withdrawal of the nucleus from the cells caused structural damage, outside-out patches were used to seal the electrodes if the cells were to be subsequently examined immunohistochemically. The contents of the electrode were forced into a microtube and reverse transcribed, amplified, low molecular weight components removed and all of the product *in vitro* transcribed as described for Example V. After Dnase treatment the RNA was reverse transcribed using Cy3 or Cy5 labelled dCTP prior to application to the microarrays.

Microarray Synthesis

Custom synthesised amine-modified oligonucleotide probes (probes) were purified in desalting columns to remove amine contaminants. The probes were prepared to a final concentration of 10-25 nmole/ml in 1X Surmodics Printing Buffer, containing 150 mM sodium phosphate, pH 8.5 (SurModics Inc, USA). The probe solution was printed

on 3D-Link Activated Slides (SurModics Inc, USA), and stored overnight in a saturated NaCl chamber. Printed slides were stored at room temperature. The microarrays contained probes capable of recognising the bacterial sequences which were included in the patch electrode buffer. These served to ensure that successful amplification had occurred. In addition 3 probes from the Dengue virus genome were included as negative controls. The arrays contained a total of 510 oligonucleotide probes, recognising 141 different transcripts, each transcript being recognised by 3 or more separate probes.

Microarray hybridisation

Slides were exposed to 15 ml SurModics Blocking Solution (50 mM ethanolamine, 0.1 M Tris, pH 9) with 0.1 % SDS at 50°C for 15 minutes. Slides were rinsed twice with water, and washed with 15 ml 4X SSC / 0.1 % SDS prewarmed to 50°C for 40 minutes on a shaker. Slides were washed with water, and centrifuged at 800 rpm for 3 minutes. Labelled cDNA (target) hybridisation mixture was heated for 2 minutes in a boiling water bath, spun briefly to cool, and 2.5 l of target added per cm² of coverslip. Slides were placed in a humidified incubator overnight. Slides were removed from the incubation chamber and successively washed with 4X SSC for 30 seconds, 2X SSC / 0.1 % SDS for 5 minutes, 0.2X SSC for 1 minute and 0.1X SSC for 1 minute. Slides were spun to dry and scanned.

Gene expression analysis of single cells after cDNA amplification using microarrays.

cDNA amplification by embodiment 3 was used to assess the expression of a large number of genes in 4 striatal cholinergic neurons, the aim being the detection of both low and high abundance transcripts. In any analysis of gene expression at the single cell level problems are encountered with low abundance transcripts and with the non detection of some mRNAs in subpopulations of cells. This has been discussed in Surmeier et al (1996) J. Neurosci. 16, 6579-6591 and Richardson et al. (2000) J. Neurochem 74, 839-846.

Currently it is accepted that the number of cells in which a transcript is detected is related to the abundance of the transcript i.e. the more often a transcript is detected in individual cells the more abundant is the mRNA. Thus in any study of an apparently homogeneous population of cells, some low abundance transcripts may be detected in only a subpopulation of cells. For example, many GABA_A receptor subunit mRNAs were detected in less than 100% of the cholinergic neurons tested by Yan and Surmeier (1997), suggesting that these transcripts were expressed either at low abundance in all the cells, or only in a specific subpopulation of cells. In the former case, more sensitive techniques will reveal a higher proportion of cells as positive for given transcripts, whereas in the latter there will be little change in the % of cells positive for a given transcript.

Table 1 shows some of the genes detected i.e. those whose expression in these cells had been previously characterised, and that the bacterial positive controls were detected but not the viral negative controls. All the housekeeping mRNAs are expected to be expressed in all cholinergic neurons. The neuronal markers dynorphin, enkephalin and PPTA are markers for non cholinergic neurons in the striatum, and lipoprotein lipase for endothelial cells.

Table 1 also shows that the use of embodiment 3 increased the number of cells (compared to previous estimates) in which the Voltage sensitive Na channel $\alpha 6$, trkC and NK3R mRNAs were detected, showing the ability of this method to detect low abundance transcripts in single cells.

In addition a number of mRNAs not previously suspected to be expressed in these cells were detected including somatostatin (SST), mAChR3 and 5, SUR2 and the D3 and D4 receptors, again showing the high sensitivity of this method.

In contrast, many of the GABA receptor subunit mRNAs were only detected in a proportion of the cells, suggesting that subpopulations of these cells may exist which express different complements of GABA receptor subunits, as suggested by Yan and Surmeier (1997). Other references in table 1 are: Yan & Surmeier (1996), Yan et al., (1997) and Tallaksen-Greene et al (1998).

Table 1: List of mRNAs detected in 4 single cholinergic neurons using embodiment 3 followed by hybridization to microarrays. The percentage of cells expected (e.g. housekeeping mRNAs are expected to be expressed in all cells) or previously shown by other methods, is shown in the third column (% positive cells) with the appropriate reference in the second column. The percentage of cholinergic cells in which the corresponding mRNAs were detected after embodiment 3 and microarray analysis is shown in column 4 (% positive cells by embodiment III).

Table 1

Housekeeping genes	Reference	% +ve predicted	% +ve by embodiment 3
Ribosomal L18	Expected	100	100
GAPDH	Expected	100	75
G6PDH	Expected	100	75
NS Enolase	Expected	100	100
α tubulin	Expected	100	75
Heavy Neurofil	Expected	100	100
Neurofil 68	Expected	100	100
β actin	Expected	100	75
MAP2	Expected	100	75
Neuronal cell markers			
Dynorphin	Expected	0	0
Enkephalin	Expected	0	0
SST	Expected	0	50
Parvalbumin		Unknown	50
Calretinin		Unknown	75
PPTA	Expected	0	0
GAD67	Richardson et al (2000)	28	75
GAD65	Expected	28	25
Lipoprotein lipase	Expected	0	0
Glutamate receptor subunits			
NR1	Richardson et al (2000)	100	100

NR2A	Richardson et al (2000)	50	75
NR2B	Richardson et al (2000)	20	50
NR2C	Richardson et al (2000)	0	100
NR2D	Richardson et al (2000)	80	100
GluR1	Richardson et al (2000)	30	75
GluR2	Richardson et al (2000)	40	50
GluR3	Richardson et al (2000)	30	50
GluR4	Richardson et al (2000)	20	25
mGluR1	Tallaksen-Greene et al (1998)	89	25
mGluR5	Tallaksen-Greene et al (1998)	65	50

GABA receptor subunits

GABA α 1	Yan & Surmeier (1997)	Present	50
GABA α 2	Yan & Surmeier (1997)	Present	25
GABA α 3	Yan & Surmeier (1997)	73	100
GABA α 4	Yan & Surmeier (1997)	92	75
GABA β 1	Yan & Surmeier (1997)	100	50
GABA β 2	Yan & Surmeier (1997)	39	25
GABA β 3	Yan & Surmeier (1997)	77	50
GABA γ 1	Yan & Surmeier (1997)	62	0
GABA γ 2	Yan & Surmeier (1997)	100	75
GABA γ 3	Yan & Surmeier (1997)	77	50

Growth factor receptors

trkA	Richardson et al (2000)	100	75
trkB	Richardson et al (2000)	70	100

trkC	Richardson et al (2000)	10	75
Sulphonylurea receptors			
SUR1	Lee et al (1998)	100	75
SUR2	Lee et al (1998)	0	100
Voltage sensitive Ca channel subunits			
$\alpha 1$ A	Yan & Surmeier (1996)	60	75
$\alpha 1$ B	Yan & Surmeier (1996)	90	25
$\alpha 1$ C	Yan & Surmeier (1996)	50	100
$\alpha 1$ D	Yan & Surmeier (1996)	50	100
$\alpha 1$ E	Yan & Surmeier (1996)	50	50
G protein coupled receptors			
mAChR1	Yan & Surmeier (1996)	30	100
mAChR3	Yan & Surmeier (1996)	0	25
mAChR4	Yan & Surmeier (1996)	90	100
mAChR5	Yan & Surmeier (1996)	0	100
Adenosine A1R	Richardson et al (2000)	60	100
Adenosine A2AR	Richardson et al (2000)	45	75
Adenosine A2BR	Richardson et al (2000)	30	50
Adenosine A3R	Richardson et al (2000)	65	0
Dopamine D1R	Yan et al (1997)	20	25

3. The method of claims 1 or 2, wherein the amplification reaction of step c) includes the steps of :

(i) obtaining single stranded DNA molecules at a temperature comprised between 85°C and 97°C;

5 (ii) annealing the single stranded DNA molecules at a temperature comprised between 55°C and 65°C; in the presence of a concentration of magnesium ranging from 1.5-6 mM.

(iii) elongating the annealed DNA molecules at a temperature comprised between 70°C and 75°C in the presence of a concentration of
10 4.5 mM Magnesium;

(iv) reiterating steps (i) to (iii) for the desired number of cycles.

4. The method of any one of claims 1 to 3, wherein the amplification of said first and second cDNA strands carried out in step c)
15 comprises between 30 and 50 amplification cycles.

5. The method according to any one of claims 1 to 4, wherein each amplification reaction cycle of step e) comprises the following steps of :

20 (i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 85°C and 95°C ;

(ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature comprised between 55°C and 75°C;

(iii) elongating the annealed DNA molecules using a
25 thermoresistant DNA polymerase at a temperature comprised between 65°C and 75 °C;

(iv) reiterating steps (i) through (iii) for the desired number of cycles desired.

6. The method of any one of claims 1 to 5, wherein the further
30 set of amplification reaction cycles carried out in step e) comprises between 10 and 40 cycles.

7. The method of claim 5 or 6, wherein the amplification reaction of step e) is performed in the presence of magnesium
35 concentration ranging from 1,5 to 4,5 mM.

8. The method according to any one of claims 1 to 7, wherein said method comprises an additional amplification step following step f) which comprises submitting at least a part of the high molecular weight
5 DNA molecules separated at step f) to a further amplification reaction using at least a portion of the first heel sequence and at least a portion of the second heel sequence as the primers.

9. The method of any one of claims 1 to 8, wherein step g)
10 comprises any one of the following methods :

(i) detection of the sequences of interest with specific oligonucleotide probes;

(ii) amplification of the sequences of interest with specific oligonucleotide primers;

15 (iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or

(iv) in vitro RNA transcription, either for hybridization assays or for further reverse transcription optionally using unlabelled or labeled substrate followed by gene specific PCR or hybridization.

20

10. The method according to any one of claims 1 to 9, wherein the first heeled primer population consists of a population of nucleic acid sequences each comprising, from 5'end to 3'end :

25 (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample or to the first strand cDNA molecules obtained at step a);

(ii) an oligo dT sequence of 15 to 25 nucleotides in length; and

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof,
30 wherein substantially every possible variable sequence combination is found in said first heeled primer population.

11. The method according to claim 10, wherein said first heeled primer comprises a RNA polymerase binding site such as the T7
35 promoter.

12. The method according to any one of claims 1 to 11, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5'end to 3' end :

- 5 (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a);
- (ii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of
- 10 4 to 7 nucleotides is found in said second heeled primer population ; and
- (iii) a second variable nucleotide sequence that was calculated to hybridize on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

15 13. The method according to claim 11, wherein the heel sequence consists of the nucleic acid sequence 5'-CTGCATCTATCTAATGCTCC-3'.

20 14. The method according to claim 12 or 13, wherein said second heeled primer comprises a RNA polymerase binding site such as the T7 promoter.

25 15. A method to increase the number of nucleotide sequences corresponding to the mRNA species present in a sample, wherein said method comprises the steps of :

- a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;
- b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population, wherein
- 30 each of the primers of said second heeled primer population contains a rare cleavage site in particular a rare restriction site located at or close to the 3'end of its heel sequence;
- c) amplifying the first and second cDNA strands resulting from step b) over a number of amplification cycles with :

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer,

5 d) incubating the product obtained at step c) with at least one restriction enzyme that specifically recognizes the cleavage site in particular a rare restriction site included in the heel sequence of the second heeled primer;

e) diluting the product of step d) to obtain a diluted cDNA solution containing a cDNA concentration which is between about 2 and 100 times inferior to the cDNA concentration of the product of step c);

f) adding a thermoresistant DNA polymerase to the diluted sample of step e) and performing a further set of amplification reaction cycles without adding further nucleic acid primer; and

15 g) confirming the presence of at least one nucleic acid sequence contained in the reaction mixture obtained at step f).

16. The method according to claim 15, wherein the amplification reaction of step c) is performed under low stringency hybridization conditions.

20

17. The method of claim 15 or 16, wherein the amplification reaction of step c) includes the following steps of :

(i) obtaining single stranded DNA molecules at a temperature comprised between 85°C and 97°C ;

25 (ii) annealing the single stranded DNA molecules at a temperature comprised between 45°C and 65°C ;

(iii) elongating the annealed DNA molecules at a temperature comprised between 70°C and 75°C in the presence of a concentration of 4.5 mM Magnesium;

30 (iv) reiterating steps (i) to (iii) for the desired number of cycles.

18. The method of any one of claims 15 to 17, wherein the amplification of said first and second cDNA strands carried out in step c) comprises between 30 and 50 amplification cycles.

35

19. The method according to any one of claims 15 to 18, wherein the first heeled primer population consists of a population of nucleic acids comprising, from 5'end to 3'end :

(i) a heel sequence of 15 to 22 nucleotides in length which is
5 not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 25 nucleotides in length;

(iii) a variable sequence of 2-4 nucleotides in length that can
hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof,
10 wherein substantially every possible variable sequence combination is found in said first heeled primer population.

20. The method according to claim 19, wherein the variable sequence of 2 to 4 nucleotides is selected among the following variable
15 nucleotide sequence : 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from A, T, C or G.

21. The method according to any one of claims 15 to 20, wherein the second heeled primer population consists of a population of
20 nucleic acid sequences each comprising, from 5'end to 3' end :

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a) and wherein the heel sequence includes the nucleotide sequence of a rare cleavage site
25 in particular a rare restriction site located at its 3'end;

(ii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of 4 to 7 nucleotides is found in said second heeled primer population ; and

(iii) a second variable nucleotide sequence that was calculated
30 to hybridize on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

22
23. The method according to claim 21, wherein the heel sequence consists of the nucleic acid sequence 5'-
35 CTGCATCTATCTAGTACGCGT-3'.

23. The method according to claim 21 or 22, wherein said second variable sequence is chosen from the group of sequences consisting of 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3', such that each of said second variable sequence is found in said second heeled primer population.

24. The method according to any one of claims 15 to 23, wherein the first heeled primer includes the sequence of a rare cleavage site in particular a rare restriction site located at the 3'end of its heel sequence.

25. The method according to claim 24, wherein the cleavage site in particular a rare restriction site of said first heeled primer is identical to the cleavage site in particular a rare restriction site of the second heeled primer.

26. The method according to claim 24, wherein the cleavage site in particular a rare restriction site of said first heeled primer is different from the cleavage site in particular a rare restriction site of the second heeled primer.

27. The method according to claim 26, wherein in step d) the DNA molecules amplified at step c) are incubated with two restriction enzymes recognizing respectively the rare cleavage site in particular a rare restriction site of the first and the second heeled primer.

28. The method according to any one of claims 15 to 27, wherein each amplification reaction cycle of step f) comprises the following steps of :

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 85°C and 95°C ;

(ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature comprised between 55°C and 75°C;

(iii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature comprised between 65°C and 75 °C;

5 (iv) reiterating steps (i) through (iii) for the desired number of cycles.

29. The method of any one of claims 15 to 28, wherein the further set of amplification reaction cycles carried out in step (f) comprises between 20 and 40 cycles.

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30. The method of claim 28 or 29, wherein the amplification reaction of step f) is performed in the presence of a 3.5 mM Magnesium concentration.

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31. The method according to any one of claims 15 to 30, wherein said method comprises a further step wherein the DNA molecules obtained at step f) having a length of less than 50 base pairs are separated from the reaction mixture.

20

32. The method of any one of claims 15 to 31, wherein step g) comprises any one of the following methods :

(i) detection of sequences of interest with specific oligonucleotide probes;

25 (ii) amplification of sequences of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or

30 (iv) in vitro RNA transcription, either for hybridization assays or for further reverse transcription using unlabeled or labeled primers or substrates followed by gene specific PCR or hybridization.

33. A heeled primer population comprising :

35 (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

- (ii) an oligo dT sequence of 15 to 25 nucleotides in length;
- (iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof, wherein substantially every possible variable sequence combination is found in said first heeled primer population.

34. The heeled primer population according to claim 33, wherein the variable sequence of 2 to 4 nucleotides is selected among the following variable nucleotide sequence : 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from A, T, C or G.

35. The heeled primer population of claim 33 or 34, wherein the heel sequence comprises the sequence of a rare cleavage site in particular a rare restriction site located at its 3' end.

36. The heeled primer population of claim 35, wherein the cleavage site in particular a rare restriction site is selected from the rare cutter group of enzymes which comprises Not1, BssHII, NarI, MluI, NruI and NaeI.

37. A heeled primer population comprising :

- (i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a) and wherein the heel sequence includes the nucleotide sequence of a rare cleavage site in particular a rare restriction site located within or close to its 3'end;
- (ii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of 4 to 7 nucleotides is found in said second heeled primer population ; and
- (iii) a second variable nucleotide sequence.

38. The heeled primer population of claim 37, wherein the cleavage site in particular a rare restriction site is selected from Not1, BssHII, NarI, MluI, NruI and NaeI.

39. The heeled primer population according to claim 37 or 38, wherein the heel sequence consists of the nucleic acid sequence 5'-CTGCATCTATCTAGTACGCGT-3'.

5 40. The heeled primer population according to any one of claims 37 to 39, which further comprises a RNA polymerase promoter site.

10 41. A kit for the amplification of the mRNA species present in a sample, wherein said kit comprises :

- (i) a first heeled primer population according to any one of claims 33 to 36; and
- (ii) a second heeled primer population according to any one of claims 37 to 40.

15

42. The kit of claim 41, which further comprises :

(iii) a first primer consisting of the heel sequence of the first heeled primer;

20 (iv) a second primer consisting of the heel sequence of the second heeled primer.

43. The kit according to claim 41 or 42, which further comprises one or several restriction enzymes that recognize the rare cleavage site in particular a rare restriction site sequence present in the heel sequence
25 of the second heeled primer.

44. The kit according to any one of claims 41 to 43 which further comprises a RNA polymerase.

30 45. A method to increase the number of sequences corresponding to the mRNA species present in a sample, wherein said method comprises the steps of :

a) reverse transcribing the mRNA species using a first heeled primer population to provide first strand cDNA species;

48. The method of any one of claims 45 to 47, wherein the amplification reaction of step c) is performed in the presence of a concentration of 4.5 mM Magnesium.

5 49. The method of any one of claims 45 to 48, wherein the further set of amplification reaction cycles carried out in step d) comprises between 30 and 50 cycles.

10 50. The method of any one of claims 45 to 49, wherein each amplification reaction cycle of step d) comprises the following steps of :

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 78°C and 95°C;

15 (ii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature comprised between 65°C and 75 °C;

(iv) reiterating steps (i) through (iii) for the desired number of reaction cycles.

20 51. The method of any one of claims 45 to 50, wherein the amplification reaction of step d) is performed in the presence of 2.5 mM Magnesium concentration.

52. The method of any one of claims 45 to 51, wherein said method comprises the additional step of :

25 f) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at step e).

30 53. The method according to claim 52, wherein said method comprises a further step wherein the DNA molecules obtained at step f) having length of less than 50 base pairs are discarded from the reaction mixture.

54. The method of claim 52 or 53, wherein step f) comprises any one of the following methods :

(i) a heel sequence of 25 to 30 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a);

5 (ii) a first variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of 15 to 25 nucleotides is found in said second heeled primer population ; and

(iii) a second variable nucleotide sequence.

10 59. The method of any one of claims 45 to 58, wherein the heel sequence of the second heeled primer comprises the sequence of a rare cleavage site in particular a rare restriction site located at the 3'end of its heel sequence.

15 60. The method according to claim 59, wherein said second heeled primer comprises a RNA polymerase binding site located downstream from said cleavage site in particular a rare restriction site.

20 61. The method of any one of claims 45 to 60, wherein the heel sequences of the first and second heeled primers comprise the sequence of a rare cleavage site in particular a rare restriction site located at the 3'end of their respective heel sequence.

25 62. The method according to claim 58, wherein the cleavage site in particular a rare restriction site sequence of the first heeled primer is identical to the cleavage site in particular a rare restriction site sequence present in the heel of the second heeled primer.

30 63. The method according to claim 61, wherein the cleavage site in particular a rare restriction site sequence of the first heeled primer is different from the cleavage site in particular a rare restriction site sequence present in the heel of the second heeled primer.

35 64. The method of any one of claims 60 to 63, wherein step e) is followed by the additional steps of :

g) incubating the DNA molecules obtained at step e) with at least one restriction enzyme that specifically recognizes the cleavage site in particular a rare restriction site included in the heel sequence of the second heeled primer;

5 h) diluting the product of step d) to obtain a diluted cDNA solution containing a cDNA concentration which is between about 2 and 100 times inferior to the cDNA concentration of the product of step d);

i) adding a thermoresistant DNA polymerase to the diluted sample of step h) and performing a further set of amplification reaction cycles without adding any nucleic acid primer; and

10 j) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at steps g), h) and i).

15 65. The method according to claim 64, wherein when the heels of the first and second heeled primers each comprise a rare cleavage site in particular a rare restriction site, the method is characterized in that in step g), the DNA molecules are incubated with restriction enzymes recognizing respectively the rare cleavage site in particular a rare restriction sites present in the heel sequence of the first and the second heeled primer.

20 66. The method according to claim 64 or 65, wherein said method comprises a further step wherein the DNA molecules obtained at step g) having length of less than 50 base pairs are separated from the reaction mixture.

25 67. The method according to any one of claims 64 to 66, wherein the number of amplification reaction cycles performed in step i) is comprised between 20 and 40.

30 68. The method of any one of claims 61 to 64, wherein step j) comprises any one of the following methods :

(i) detection of sequences of interest with specific oligonucleotide probes;

- (ii) amplification of sequences of interest with specific oligonucleotide primers; and
- (iii) cloning of the DNA molecules obtained in a replication and/or expression vector.

5

69. A method to increase the number of sequences corresponding to the mRNA species present in a sample, wherein said method comprises the steps of :

- a) reverse transcribing the mRNA species using a first heeled primer population to provide first strand cDNA species;
- b) synthesizing second cDNA strands using a second heeled primer population;
- c) amplifying said second cDNA strands resulting from step b) over a number of amplification cycles with second heeled primers
- d) amplifying the first and second strands resulting from step c) using primers selected from the group consisting of (1) a primer comprising a portion of the heel sequence of the first heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified,
- (2) a primer comprising a portion of the heel sequence of the second heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, and (3) a mixture of the primers (1) and (2), wherein the total concentration of primers ranges between 0.02 and 500 ng per reaction in the following conditions :
- (i) adding the primers to the cDNA product obtained at step c);
- (ii) obtaining single stranded DNA molecules at a temperature comprised between 80°C and 95°C;
- (iii) adding a thermoresistant DNA polymerase;
- (iv) maintaining the temperature at a range from 80°C to 95°C for a period of time comprised between 5 sec to 15 min;
- (v) annealing said single stranded DNA and elongating the annealed DNA molecules at a temperature comprised between 65°C and 75 °C;

(vi) carrying out steps (iv) and (v) for a desired number of cycles.

e) recovering the population of DNA molecules obtained at step

5 d)

70. The method of claim 69, wherein step d) further comprises the steps of amplifying the DNA molecules obtained at step d) (vi) over a number of amplification cycles with primers selected from the group
10 consisting of (a) a primer comprising a portion of the heel sequence of the first heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, (b) a primer comprising a portion of the heel
15 sequence of the second heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, and (c) a mixture of the primers (a) and (b), wherein the total concentration of primers ranges between 0.02 and 200 ng per reaction in the following conditions :

(vii) obtaining single stranded DNA molecules at a temperature
20 comprised between 80°C and 95°C;

(viii) adding a thermoresistant DNA polymerase to the single stranded DNA molecules obtained at step (vii);

(ix) annealing and elongating the single stranded DNA molecules at a temperature comprised between 65°C and 75°C.

25 (x) carrying out steps (vii) and (ix) for a desired number of cycles.

71. The method of claim 69, wherein step c) (vi) comprises between 10 and 50 amplification cycles.

30

72. The method of claim 69, wherein the amplification reaction of step c) is performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme.

73. The method of claim 69, wherein the amplification steps b) and c) are performed in the presence of a concentration of Magnesium comprised between 2 and 5 mM.

5 74. The method of claim 88, wherein the amplification step d) is performed in the presence of a concentration of Magnesium comprised between 2 and 3 mM.

10 75. The method of claim 69, wherein the respective concentration of primers at steps b) and c) range from 0.02 to 500 ng.

76. The method of claim 69, wherein the respective concentration of primers ranges from 0.02 to 90 ng.

15 77. The method of claim 69, wherein the respective concentration of primers at step d) ranges from 100 to 500 ng.

20 78. The method of claim 70, wherein step d) (x) comprises between 20 and 60 amplification cycles.

25 79. The method of claim 70, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme.

80. The method of claim 70, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium comprised between 1.5 and 4 mM.

30 81. The method of claim 80, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium comprised between 1.6 and 2.5 mM.

82. The method of claim 80, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium of 2.0 mM.

5 83. The method of claim 70, wherein the respective concentration of primers at steps d) (vii) to (x) ranges from 10 to 500 ng.

84. The method of claim 83, wherein the respective concentration of primers at steps d) (vii) to (x) ranges from 30 to 300 ng.

10

85. The method of claim 69, wherein step b) of synthesizing second cDNA strands is performed in the presence of a Magnesium concentration ranging from 3 to 5 mM.

15 86. The method of claim 85 wherein the Magnesium concentration is of 4.5mM.

87. The method of claim 69 or 70, wherein step b) of synthesizing second cDNA strands comprises the steps of :

(i) adding the primers to the cDNA product obtained at step a);
(ii) obtaining single stranded DNA molecules at a temperature
20 comprised between 80°C and 95°C

(iii) adding the DNA polymerase and the proof reading enzyme to the mixture obtained at step (ii);

(iv) maintaining the temperature of the mixture at approximately 94°C during a period of time comprised between 30 sec to 5 min

25 (v) annealing said single stranded DNA at a temperature comprised between 40°C and 72 °C;

(vi) elongating the annealed DNA molecules at a temperature comprised between 60°C and 75 °C;

30 88. The method of claim 69 or 70, wherein step c) of synthesizing second cDNA strands comprises the steps of :

(i) optionally obtaining single stranded DNA molecules at a temperature comprised between 80°C and 95°C in the presence of a thermoresistant DNA polymerase;

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 35 nucleotides in length; and

5 (iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof, wherein substantially every possible variable sequence combination is found in said first heeled primer population.

10 94. The method according to claim 93, wherein the variable sequence of 2 to 4 nucleotides is selected among the following variable nucleotide sequence : 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from A, T, C or G.

15 95. The method according to any one of claims 69 to 94, wherein the first heeled primer includes the sequence of a rare cleavage site in particular a rare restriction site.

20 96. The method according to claim 95, wherein the rare cleavage site in particular a rare restriction site is located at the 5' end of the heel sequence of said first heeled primer.

25 97. The method according to claim 95, wherein the rare cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence of said first heeled primer.

98. The method according to any one of claims 69 to 97, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5'end to 3' end :

30 (i) a heel sequence of 25 to 75 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a); and

(ii) a variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of
35 15 to 25 nucleotides is found in said second heeled primer population;

99. The method of claim 98, wherein the heel sequence of said second heeled primer comprises the sequence of a rare cleavage site in particular a rare restriction site.

5

100. The method of claim 99, wherein the sequence of a rare cleavage site in particular a rare restriction site is located at the 5' end of the heel sequence.

10

101. The method of claim 100, wherein the sequence of a rare cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence.

15

102. The method of claim 99, wherein the heel sequence of the second heel primer ranges from 25 to 35 nucleotides in length.

20

103. The method of claim 98 wherein the heel sequence of the second heeled primer ranges from 45 and 75 nucleotides in length and comprises a RNA polymerase binding site.

25

104. The method of claim 99, wherein the heel sequence of the second heeled primer ranges from 45 and 75 nucleotides in length and comprises a RNA polymerase binding site located at the 3' end of the heel sequence.

30

105. The method according to any one of claims 99 to 104, wherein step e) is followed by the additional steps of :

g) incubating the DNA molecules obtained at step e) with at least one restriction enzyme that specifically recognizes the cleavage site in particular a rare restriction site included in the heel sequence of the second heeled primer;

35

h) diluting the product obtained at the end of steps d) and g) to obtain a diluted cDNA solution containing a cDNA concentration which is between 2 and 100 times inferior to the cDNA concentration of the product of step d) or g);

i) adding a thermoresistant DNA polymerase to the diluted sample of step h) and performing a further set of amplification reaction cycles without adding any nucleic acid primer; and

5 j) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at steps g), h) and i).

10 106. The method of claim 105, wherein when the heels of the first and second heeled primers each comprise a rare cleavage site in particular a rare restriction site, the method is characterized in that in step g), the DNA molecules are incubated with restriction enzymes recognizing respectively the rare cleavage site in particular a rare restriction sites present in the heel sequence of the first and the second heeled primer.

15

107. The method according to claim 105 or 106, wherein said method comprises a further step wherein the DNA molecules obtained at steps e) and g) having length of less than 50 base pairs are separated from the reaction mixture.

20

108. The method according to any one of claims 105 to 107, wherein the number of amplification reaction cycles performed in step i) is comprised between 20 and 40.

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109. The method of any one of claims 105 to 108, wherein step j) comprises any one of the following methods :

(i) detection of sequences of interest with specific oligonucleotide probes;

30 (ii) amplification of sequences of interest with specific oligonucleotide primers; and

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector.

110. The method according to any one of claims 69 to 109, wherein the heel sequences of the first heeled primer and the second heeled primer are identical.

5 111. A heeled primer population comprising :

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 35 nucleotides in length; and

10 (iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof, wherein substantially every possible variable sequence combination is found in said first heeled primer population.

15 112. The heeled primer population according to claim 111, wherein the variable sequence of 2 to 4 nucleotides is selected among the following variable dinucleotide sequence : 5'-(A or G or C)-N₁₋₃, wherein N is a nucleotide selected from A, T, C or G.

20 113. The heeled primer population of claim 111 or 112, wherein the heel sequence comprises the sequence of a rare cleavage site in particular a rare restriction site.

25 114. The heeled primer population of claim 113, wherein the cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence.

30 115. The heeled primer population of claim 113, wherein the cleavage site in particular a rare restriction site is located at the 5' end of the heel sequence.

35 116. The heeled primer population of claim 113, wherein the cleavage site in particular a rare restriction site is selected from the rare cutter group of enzymes which comprises Not1, BssHII, NarI, MluI, NruI and NaeI.

117. The heeled primer population of any one of claims 111 to 116, wherein the heel sequence has a GC content comprised between 50 and 80%.

5

118. A heeled primer population comprising :

(i) a heel sequence of 25 to 75 nucleotides in length which is not complementary to the mRNA molecules or with first strand cDNA molecules present in a sample;

10

(ii) a first variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of 15 to 25 nucleotides is found in said second heeled primer population.

119. The heeled primer population of claim 118, wherein each primer further comprises :

15

(iii) a second variable nucleotide sequence that was calculated to hybridize on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

120. The heeled primer population according to claim 119, wherein said second variable sequence is chosen from the group of sequences consisting of 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3', such that each of said second variable sequence is found in said second heeled primer population.

20

121. The heeled primer population of claim 118, which contains the sequence of a rare cleavage site in particular a rare restriction site.

122. The heeled primer population of claim 1201, wherein the sequence of a rare cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence.

30

123. The heeled primer population of claim 121, wherein the sequence of a rare cleavage site in particular a rare restriction site is located at the 5' end of the heel sequence.

35

124. The heeled primer population of claim 121, wherein the cleavage site in particular a rare restriction site is selected from Not1, BsshII, NarI, MluI, NruI and NaeI.

5

~~125~~ 124. The heeled primer population of any one of claims 111 to 124, wherein the heel sequence has a GC content comprised between 50 and 70%.

10

126. The heeled primer population of claim 115 wherein the heel sequence ranges from 25 to 35 nucleotides in length.

15

127. The heeled primer population of claim 115 wherein the heel sequence ranges from 45 to 75 nucleotides in length and comprises a RNA polymerase binding site.

20

128. The heeled primer population of claim 127 wherein the RNA polymerase binding site is located at the 3' end of the heel sequence.

25

129. A kit for the amplification of the mRNA species present in a sample, wherein said kit comprises :

(i) a first heeled primer according to any one of claims 11 to 116;

(ii) a second heeled primer population according to claims 117 to 127.

30

130. The kit of claim 126, which further comprises :

(iii) a first primer selected from the group consisting of (a) the heel sequence of the first heeled primer and (b) a primer comprising at least 15 consecutive nucleotides of the heel sequence of the first heeled primer.;

(iv) a second primer selected from the group consisting of (a) the heel sequence of the second heeled primer and (b) a primer

comprising at least 15 consecutive nucleotides of the heel sequence of the second heeled primer.;

5 131. The kit of claim 129, wherein the heel sequences of the first heeled primer and of the second heeled primer are identical.

132. The kit of claim 130, wherein the sequences of the first – and second primers are identical.

10 133. The kit according to anyone of claims 129 to 132, which further comprises a restriction enzyme that recognizes the rare cleavage site in particular a rare restriction site sequence present in the heel sequence of the second heeled primer.

15 134. The kit according to any one of claims 129 to 133 which further comprises a restriction enzyme that recognizes the rare cleavage site in particular a rare restriction site sequence present in the heel sequence of the first heeled primer.

20 135. The kit according to any one of claims 129 to 134, which further comprises a RNA polymerase.

136. A method to increase the number of nucleotide sequences corresponding to the mRNA species present in a low quantity in a sample, comprising:

5 a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;

b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population;

10 c) amplifying said first and second cDNA strands resulting from step b) over a number of amplification cycles with the aid of a thermoresistant DNA polymerase(s) with:

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

15 (ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer,

wherein said method is characterized in that it comprises the steps of:

d') increasing the proportion of high molecular weight DNA molecules,

20 e') using or analyzing specific nucleic acid sequences present in the high molecular weight DNA molecules,

137. A method to increase the number of nucleic acid sequences corresponding to the mRNA species present in a low quantity in a sample, wherein said method comprises the steps of:

25 a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;

b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population, wherein each of the primers of said first, and/or second heeled primer population optionally contains a rare cleavage site in particular a rare restriction site located at the 3' end of its heel sequence;

30 c) amplifying the first and second cDNA strands resulting from step b) over a number of amplification cycles with:

35 (i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer;

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(54) Title: A METHOD FOR AMPLIFYING LOW ABUNDANCE NUCLEIC ACID SEQUENCES AND MEANS FOR PERFORMING SAID METHOD

(57) Abstract: The present invention relates to methods as well as to nucleic acid primers and kits containing the same for performing efficiently an amplification of nucleic acid sequences from a sample, particularly of nucleic acid sequences that are initially poorly represented in said sample.



WO 01/06004 A2

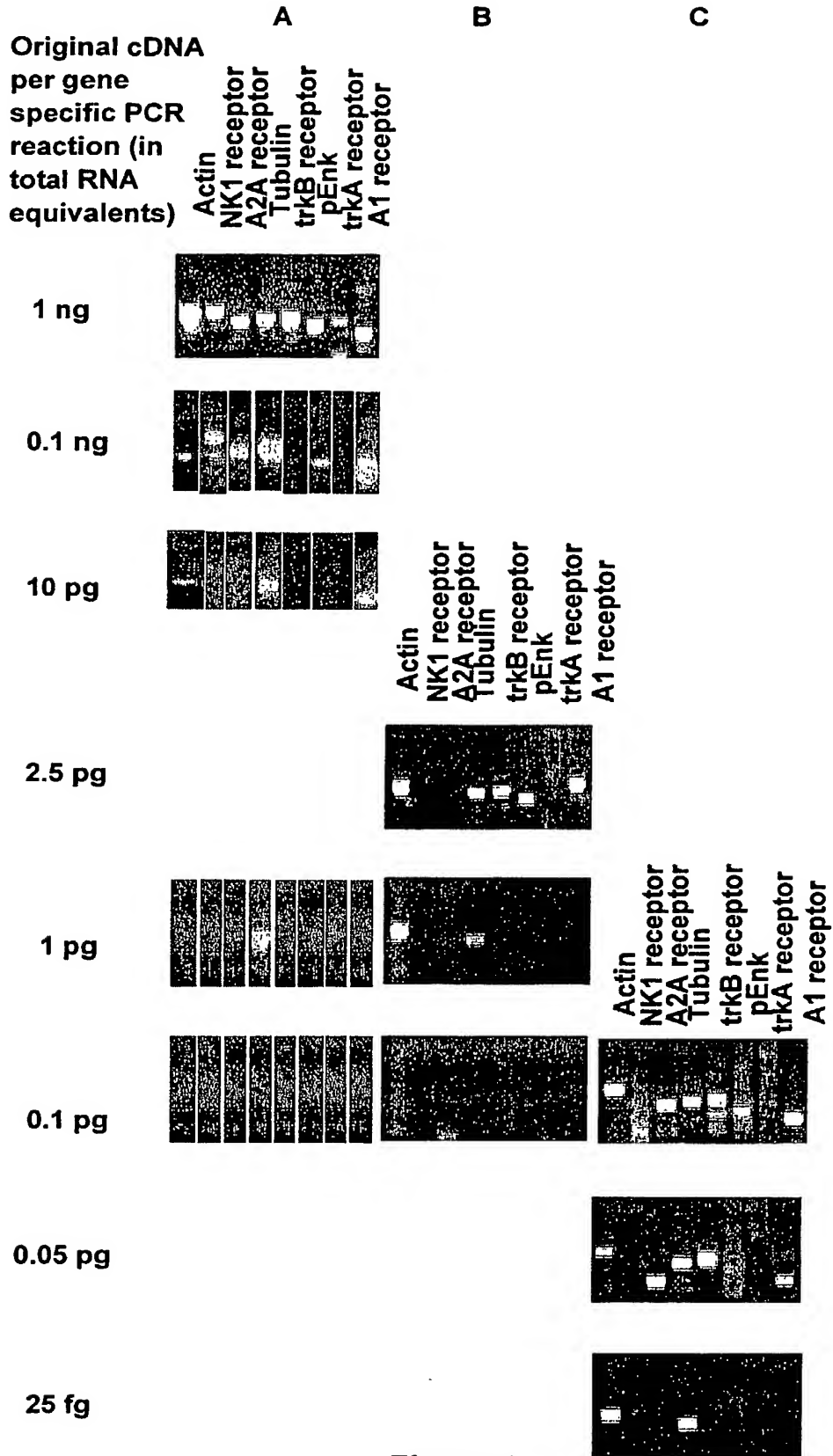


Figure 1

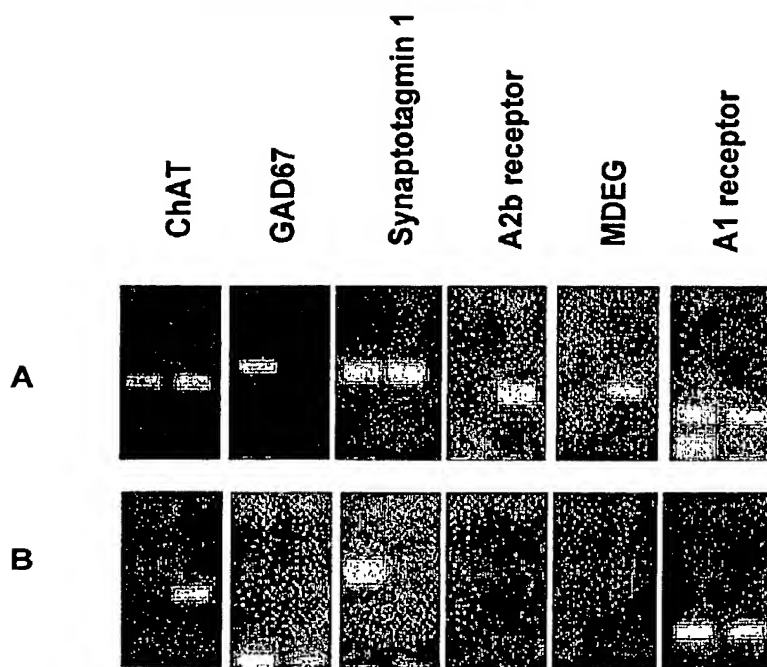
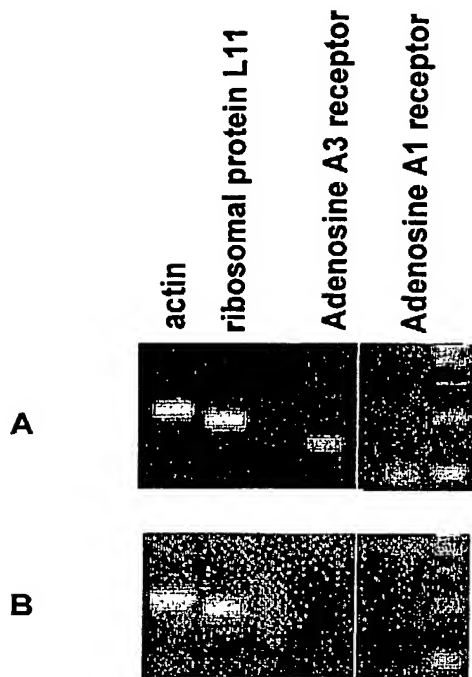


Figure 2

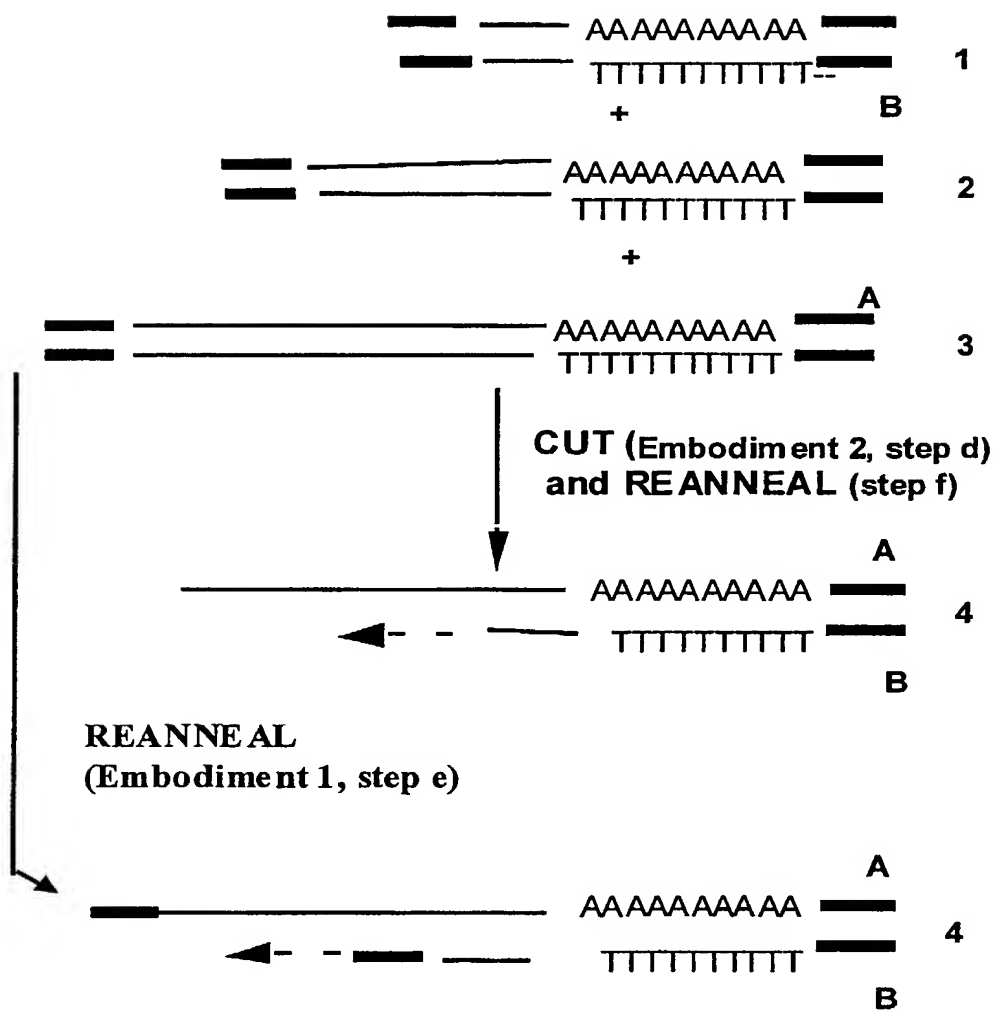


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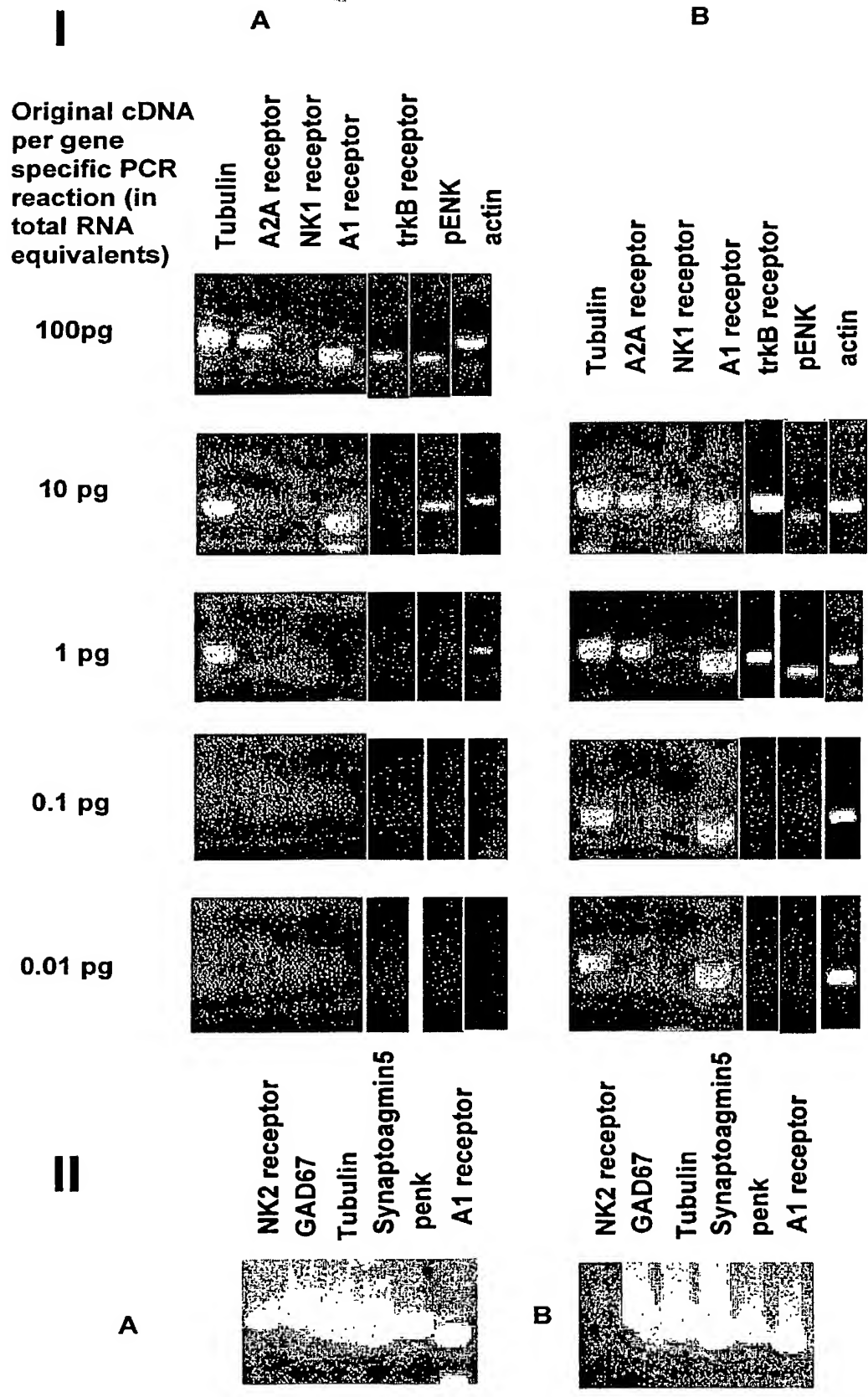


Figure 4

20-019,904

III

cDNA per gene
specific PCR reaction
(in total RNA
equivalents

1 ng cDNA

0.006% of 1
ng cDNA,
amplified to
step h)

0.006% of 1
ng cDNA, No
amplification

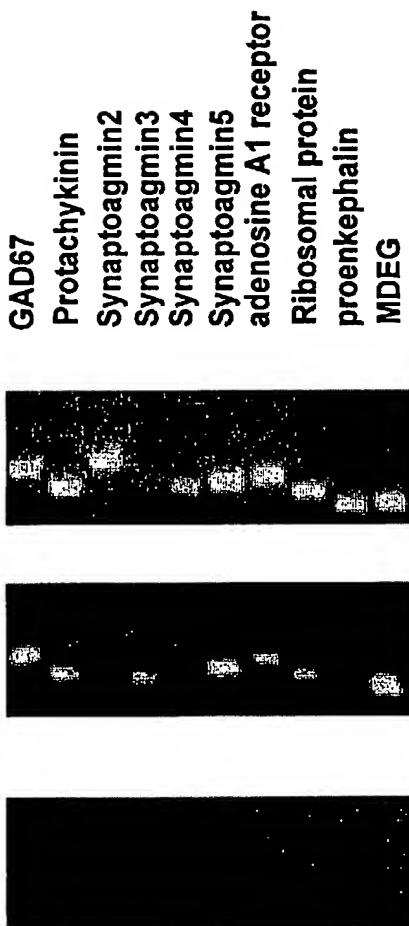


Figure 4 (CONTINUED)

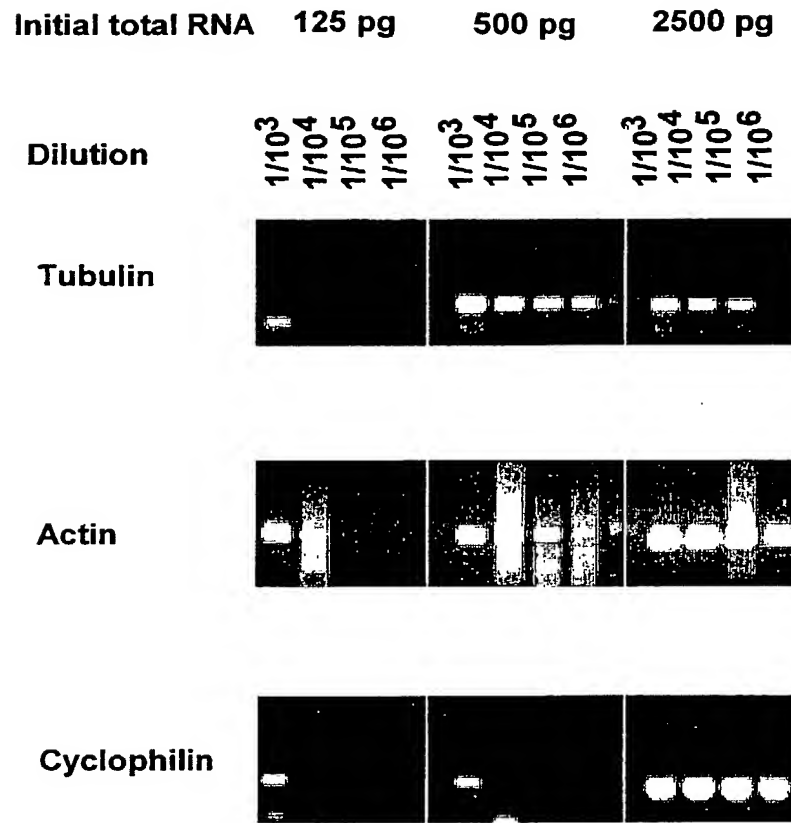


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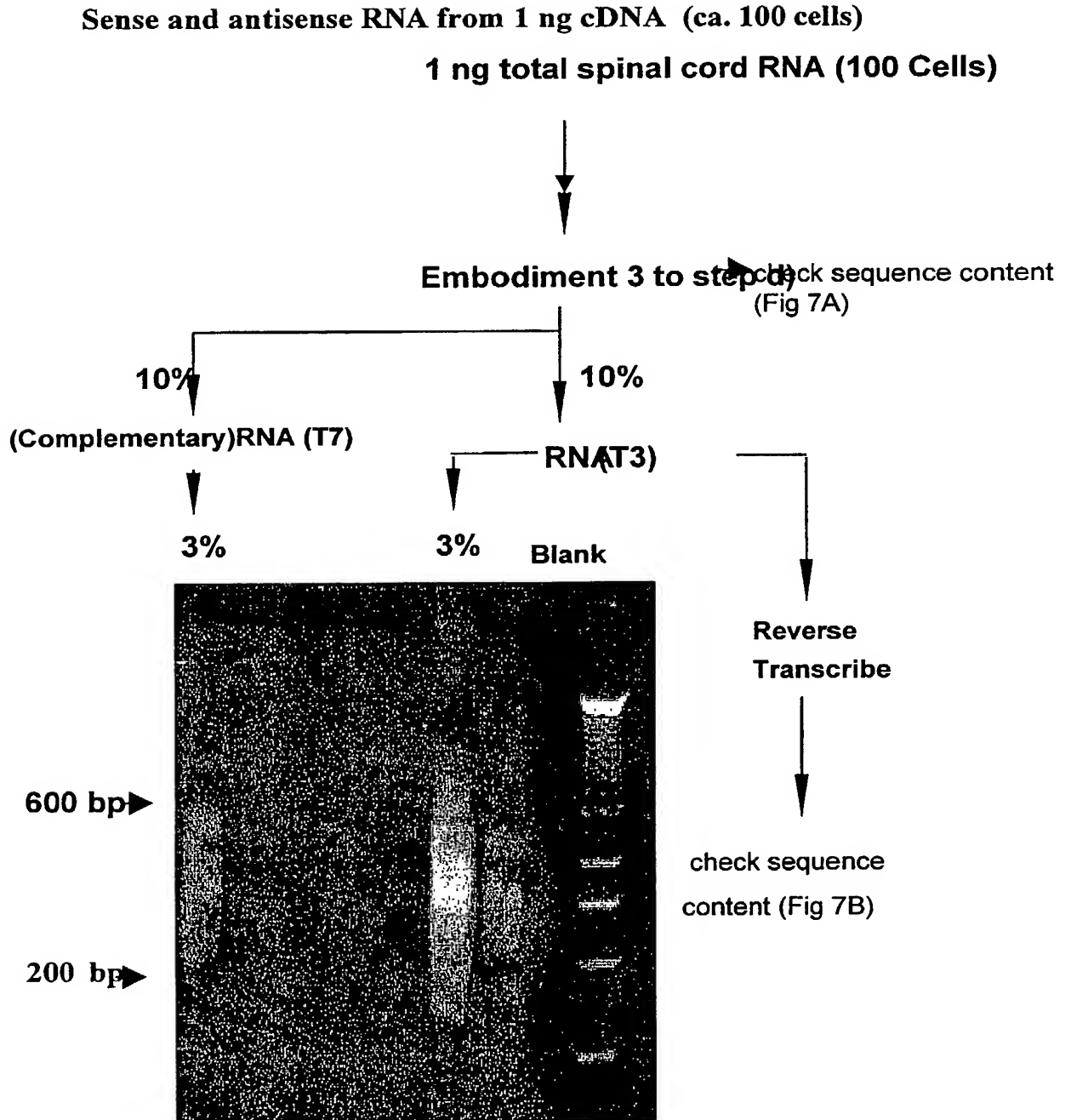


Figure 6

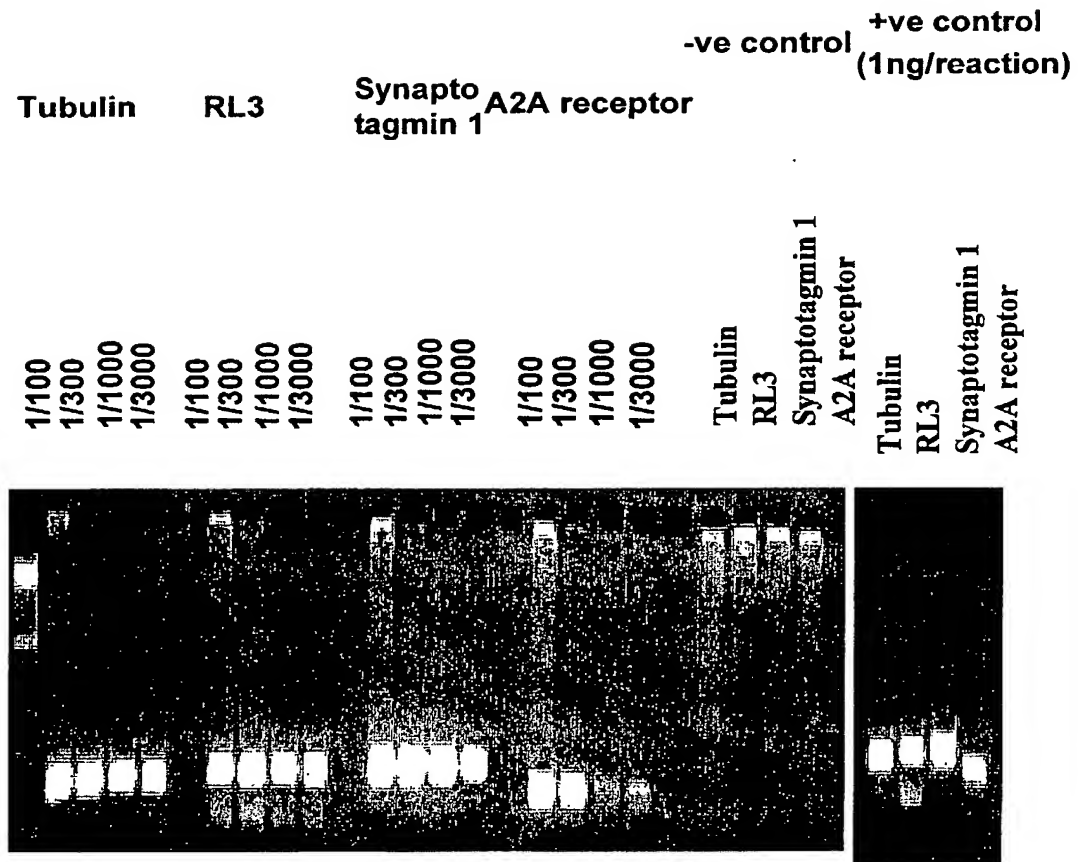


Figure 7A

10-019, 906

Dilution 1/10 ⁴		1/10 ³		+ve control	
Per gene specific reaction		(1 ng cDNA/gene specific reaction)		PCR blanks	
A2A receptor		A2A receptor		A2A receptor	
Tubulin		Tubulin		Tubulin	
Ribosomal protein L3		Ribosomal protein L3		Ribosomal protein L3	
Synaptotagmin 1		Synaptotagmin 1		Synaptotagmin 1	

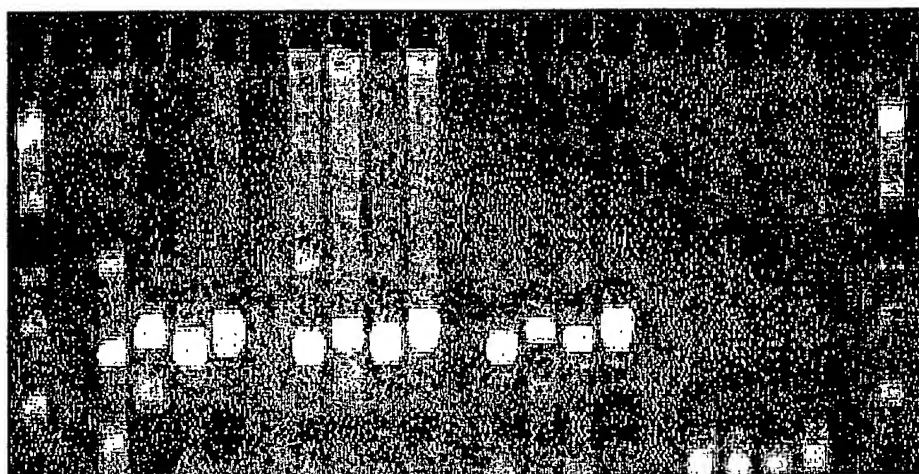


Figure 7B

USA

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of subject matter which is claimed and for which a patent is sought on an invention entitled

A METHOD FOR AMPLIFYING LOW ABUNDANCE NUCLEIC ACID SEQUENCES AND MEANS FOR PERFORMING SAID METHOD

the specification of which ☐ is attached hereto or

☒ was filed on 19 JUL 2000 as United States Application Number or PCT International Application Number PCT/EP00/06887 and was amended on 05 OCT 2001 (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for a patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application Number(s)	Country	Foreign Filing Date	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
60/144666	US	19 JUL 1999	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
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As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: David R. Saliwanchik, Reg. 31,794; Jeff Lloyd, Reg. 35,589; Doran R. Pace, Reg. 38,261; Christine Q. McLeod, Reg. 36,213; Jay M. Sanders, Reg. 39,355; James S. Parker, Reg. 40,119; Jean E. Kyle, Reg. 36,987; Frank C. Eisenschenk, Reg. 45,332; Seth M. Blum, Reg. 45,489; Glenn P. Ladwig, Reg. 46,853; Margaret Efron, Reg. 47,545 and Jon Michael Gibbs, Reg. 47,594

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Gainesville, FL 32606-6669
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C 1001 and that such willful false statements may jeopardise the validity of the application or any patent issued thereon.

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4/1/02

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Date of signature

22/2/02

SEQUENCE LISTING

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5 <120> A method for amplifying low abundance nucleic acid sequences and means for performing said method.

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